DEVELOPMENT OF A STRATEGY FOR GENETIC TRANSFORMATION OF PLANT MITOCHONDRIA.

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... 'Let's look for dragons' I said to Pooh.
'Yes, let's', said Pooh to me.
We crossed the river and found a few-
'Yes, those are dragons all right,' said Pooh.
'As soon as I saw their beaks I knew,
Thats what they are,' said Pooh, said he.
'Thats what they are', said Pooh. ...

Us Two by A.A. Milne.

When I set out for Lyonnesse,
A hundred miles away,
The rime was on the spray,
And starlight lit my lonesomeness
When I set out for Lyonnesse
A hundred miles away.

What would bechance at Lyonnesse
While I should sojourn there
No prophet durst declare,
Nor did the wisest wizard guess
What would bechance at Lyonnesse
While I should sojourn there. ...

Thomas Hardy.

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ABSTRACT.

Mitochondria of higher plants, like those of other organisms, contain their own genome. The organisation, evolution, and coding capacity of higher plant mitochondrial genomes have been the subject of detailed descriptive analyses. Progress towards a mechanistic understanding of plant mitochondrial genomes has been hampered by the lack of *in vitro* assays and informative mutations such as those that have provided effective experimental systems for the analysis of fungal and mammalian mitochondrial genomes. This thesis describes an attempt to establish a procedure for genetic transformation of higher plant mitochondria which, it was hoped, would facilitate experimental analysis and perhaps beneficial modification of their genomes.

The strategy adopted for recovering mitochondrial transformants involved the construction *in vitro* of chimaeric chloramphenicol acetyltransferase (CAT) genes that were intended to confer selectable chloramphenicol resistance to plant cells, but only if they were situated in the mitochondria. The work involved two parts.

Firstly, reliable procedures were established for the recovery of chloramphenicol resistant nuclear transformants using *Nicotiana tabacum* and several different chimaeric CAT genes. Nuclear transformants were recovered from protoplasts using direct DNA transfer and *Agrobacterium tumefaciens*, and from leaf disc transformation. Chloramphenicol resistant calli were recovered with 10 to 20% of the frequency with which kanamycin resistant calli were recovered following transformation with chimaeric *nptII* genes. In contrast to previous reports these results show that chimaeric CAT genes can be used effectively to recover transformed plant cells, providing a new selectable marker for plant transformation.

Secondly, based on the current understanding of plant mitochondrial gene expression, which is discussed, I have designed and constructed transformation vectors that are intended to allow synthesis of CAT in mitochondria of *N. tabacum*. A CAT gene (*cat*) from *Proteus mirabilis* was chosen as it does not contain the codon CGG which may be translated as tryptophan in plant mitochondria rather than arginine as in the standard genetic code. The coding sequence of this gene was fused to the upstream sequence and first five codons of *coxI* from normal and CMS-S maize mitochondria, and to sequence from upstream of *atp9*-1 from *Petunia hybrida* mitochondria. These mitochondrial DNA sequences are intended to provide *cat* with the sequences necessary to promote its transcription and translation in mitochondria of *N. tabacum*.

Additional sequence from around *atp9* of *N. tabacum* and *atp9*-1 of *P. hybrida* has been included in several transformation vectors to provide sequence homology with *N. tabacum* mitochondrial DNA. Recombination between these homologous sequences would lead to insertion of the transforming DNA into the mitochondrial genome where it may be maintained. Some of these constructs contain a potential transcription terminator or processing site from *N. tabacum* mitochondria. Ti plasmid derivatives have been constructed for use with *A. tumefaciens*.

No evidence was found for expression of *cat* in the nuclei of *N. tabacum* cells following stable transformation with the mitochondrial transformation vectors, or in transient expression analyses in protoplasts. If these vectors are active in mitochondria, it appears that the desired specificity of *cat* expression has been achieved. In an alternative approach, the tryptophan codons of a second CAT gene have been converted to CGG in order that active CAT can be synthesised only if the genetic code proposed for higher plant mitochondria is used. Transformation vectors similar to those described above have been constructed with this modified CAT gene.

Mitochondrial transformation experiments have been initiated. These have employed direct DNA transfer, leaf disc transformation and cocultivation of protoplasts with *A. tumefaciens*. Preliminary results are discussed, and analysis of the first potential transformants has begun. The strategy adopted above, and alternatives are critically assessed.

Abbreviations.

1', 2'	1' and 2' genes from *A. tumefaciens* T-DNA.
2,4-D	2,4-dichlorophenoxyacetic acid
3'	3' carbon of a nucleotide or nucleoside, hydroxy terminus of a nucleic acid backbone, towards the 3' carbon.
5'	5' carbon of a nucleotide or nucleoside, hydroxy terminus of a nucleic acid backbone, towards the 5' carbon.
35S	promoter of the 35S cauliflower mosaic virus transcript.
ATP	adenosine-5'-triphosphate
atpA, atp6, atp9	mitochondrial genes encoding subunits α, 6 and 9 of the ATP synthase complex
bisacrylamide	N, N'-methylenebisacrylamide
%	percent
ANT	adenine nucleotide translocator
ATPB	β subunit of the F1-F0 ATP synthase
BAP	6-benzylaminopurine
BSA	bovine serum albumin
bp	base pairs
CAT	chloramphenicol acetyltransferase
cat	the chloramphenicol acetyltransferase gene
cm	centimetre
CMS (S,T,C)	cytoplasmic male sterility (USDA, Texas, Charrua)
cob	mitochondrial gene encoding apocytochrome b
COXI	cytochrome oxidase subunit I polypeptide
coxI, coxII, coxIII	mitochondrial genes encoding subunits 1, 2 and 3 of the cytochrome oxidase complex
CAM	crassulaceaean acid metabolism
CaMV	cauliflower mosaic virus
CMV	cucumber mosdaic virus
CTAB	cetyltrimethylammonium bromide
°C	degrees centigrade
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
DCCD	N,N'-dicyclohexylcarbodiimide
DEAE	diethylaminoethyl

DNA	deoxyribonucleic acid
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid (disodium salt)
FAD	flavine adenine dinucleotide
FRG	Federal Republic of Germany.
g	gram
GmbH	Geselschaft mit beschrennkter Haftung
GTP	guanosine-5'-triphosphate
Hants.	Hampshire.
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
IPTG	isopropyl β-D-thiogalactoside
K3	protoplast culture medium, Wullems *et al.* (1981)
K3 0.1	K3 medium with sucrose adjusted to 0.1 M
K3 0.4	K3 medium with sucrose adjusted to 0.4 M
kb	kilobase (pair(s))
KV	kilovolt
l	litre
LB	Luria broth, described in section 2.2.1
Ltd.	limited
LTE	tris-EDTA buffer, defined in section 2.2.1
µCi	microcurie
µF	microfarad
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometre
µW	microwatt
M	molar
MES	2-[N-morpholino]ethanesulphonic acid
mA	milliamperes
mg	milligram
ml	millilitre
mM	millimolar
mm	millimetre
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
nm	nanometre
ng	nanogram

NAA	α-naphthaleneacetic acid
NAD	nicotinamide adenine dinucleotide
NADH	β-dihydronicotinamide adenine dinucleotide
nad1	mitochondrial treading framereading frame encoding a protein with homology to part of NAD1
nad3, nad5	mitochondrial genes encoding NAD3 and NAD5
NAD1	subunit 1 of the NADH : ubiquinone oxidoreductase complex
NAD3, NAD5	subunits 3 and 5 of complex I, the NADH:ubiquinone oxidoreductase complex
NCS	non-chromosomal stripe
NOS	nopaline synthase gene
N-terminal	amino-terminal
N-type	normal, fertile
mOsm	osmolality, milliosmol/kg
ORF	open reading frame
PEG	polyethylene glycol (sometimes plus molecular weight)
plc.	public limited company.
pH	negative log of the hydrogen ion concentration
Rf	nuclear fertility restorer gene
RNA	ribonucleic acid
rpm	revolutions per minute
rps12, rps13 , rps14	reading frames encoding a polypeptide homologous to subunits 12, 13, and 14 of the small ribosomal subunit of *E. coli*
rRNA	ribosomal ribonucleic acid
S	Svedberg units
SDS	sodium dodecyl sulphate
SSC	standard saline citrate, defined in section 2.4.2.2
STIR	S plasmid terminal inverted repeat
S-type	maize cytoplasm showing CMS-S sterility
TAE	electrophoresis buffer, defined in section 2.2.3
TEA	electrophoresis buffer, defined in section 2.2.3
TBE	tris-borate electrophoresis buffer, Manniatis *et al.* (1982)
T-DNA	DNA transferred from *A. tumefaciens* to plant cells
TEMED	N,N,N',N'-tetramethylethylene-diamine
TCA	tricarboxylic acid
TIR	terminal inverted repeat
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid

USA	United States of America
U.V.	ultraviolet
V	volts
v/v	volume per volume (given as a percentage)
W	Watt
w/v	weight per volume (given as a percentage)
Xg	average maximum relative gravitational force
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside
YT	bacterial growth medium defined in section 2.2.1

CHAPTER 1.

INTRODUCTION.

This thesis concerns two distinct areas of biology; mitochondria, and genetic transformation. Mitochondria are subcellular structures specialised for the provision of energy to the rest of the cell through the process of respiration. Genetic transformation is a process by which an individual organism acquires new inheritable characteristics through the acquisition of new genetic material; this process has been exploited as an experimental technique of great value in investigating the molecular genetics of a variety of organisms. Mitochondria contain their own genetic system, but in only yeast cells is the mitochondrial genetic system open to analysis by genetic transformation. The work described in this thesis represents an attempt to apply the techniques of genetic transformation to the mitochondrial genetic system of higher plant cells.

This chapter initially provides a general introduction to the relevant aspects of plant mitochondrial biology and then a summary of the process of genetic transformation and its potential applications. This is followed by a more detailed discussion of mitochondrial genetic systems, particularly those of higher plants, and a review of the techniques currently available for genetic transformation of plant cells. Finally several instances of natural and experimental gene transfer to mitochondria and other intracellular compartments are discussed.

1.1 Mitochondria.

Eukaryotic cells can perform a variety of metabolic tasks with high efficiency owing to the distribution of functions amongst several specialised subcellular organelles. These are delineated by intracellular membranes. Each organelle contains a subset of the cellular protein and, in some cases RNA and DNA, that is specifically required for its particular role within the cell. Mitochondria are one type of such organelle, being found in most but not all eukaryotes (Cavalier-Smith 1987). They were first discovered in animal cells in 1882, and in plant cells in 1904 (Douce 1985). Their function is primarily biochemical, contributing to many vital metabolic processes; however, as they contain DNA that is essential for proper cellular function, they have in addition an important genetic role. In plant cells, the cellular genome is divided disproportionately

between three organelles, the nucleus, mitochondrion, and chloroplast; the latter two contain only a small percentage of the total cellular genome. The structure and function of mitochondria are described below.

The central conserved function of mitochondria from all organisms is in respiration. Respiration is the process of complete oxidation of carbohydrate to carbon dioxide and water using atmospheric oxygen in the final step. The energy released from respiration and the metabolism of other cellular organic compounds is converted in mitochondria into the useful phosphate bond energy of adenosine triphosphate.

Mitochondria are bounded by two membranes which are crucial to their function. The membranes are concentric and thus enclose two aqueous phases; a matrix densely packed with proteins, and a sparsely packed intermembrane space. The outer membrane defines the boundary of the organelle but, unlike the inner one, presents no barrier to the diffusion of small molecules. Glycolysis outside the mitochondrion, and the TCA cycle in the matrix generate reduced forms of cofactors NAD and FAD. The inner membrane carries an array of four different oligomeric protein complexes that transfer electrons from the reduced cofactors to molecular oxygen and in so doing translocate protons from the matrix into the intermembrane space. A concentration gradient of protons is thereby established across this membrane, and the energy released in returning them to the matrix is converted into phosphate bond energy by the F1-F0 ATP synthase (Lehninger 1975). The inner membrane is folded into numerous invaginations, called cristae, which increase its surface area such that it can exceed twice that of the cell (Douce 1985).

The structure of mitochondria is described in Douce (1985). Though the term mitochondrion is derived from the Greek words mitos, meaning thread, and chondrion, meaning granule, in plant cells mitochondria assume a variety of forms ranging from filaments to spheres. The most common shape is a rod about 0.5 μm in diameter and up to 2 μm long, with hemispherical ends.

In the Chlorophyta *Chlorella, Chlamydomonas* and *Euglena,* and the Ascomycete *Saccharomyces cerevisiae,* electron microscopic examination of serial sections through a cell revealed that what appear in single sections to be numerous discrete mitochondria in fact form a single reticulate structure throughout the cell. Similar studies have not been performed with higher plant cells. Branched mitochondria are infrequently observed in higher plant cells, and time lapse cinematography has revealed that mitochondria are mobile within the streaming cytoplasm, and able within a few seconds to change their shape, to fuse or to divide (Honda *et al.* 1966). These observations argue against higher plant mitochondria being permanently discrete entities, and against a fixed reticulate structure, but rather favour a dynamic fluid model. The genetics of mitochondrial inheritance, discussed in Chapter 5, have led Lonsdale *et al.* (1988) to similar

conclusions describing the intracellular organisation of these organelles as panmictic.

Douce (1985) estimated that exponentially growing sycamore cells contain about 250 mitochondria, occupying 0.7 % of the total cell volume, and 7 % of the cytoplasmic volume. This is less than in animal cells; however metabolically active cells such as secretory or companion cells have far more mitochondria, approaching the numbers found in animal cells.

It has been proposed that mitochondria are derived from endosymbiotic prokaryotes that have become degenerate and are no longer capable of free existence (reviewed by Gray and Doolittle 1982); the contrary hypothesis, that intracellular subdivision generated nuclei and mitochondria (reviewed in Mahler and Raff 1975), is less widely accepted. The form of the cristae varies in mitochondria from different kingdoms prompting Cavalier-Smith (1986) to propose several distinct endosymbiotic events to have occurred; this has been supported by analyses of ribosomal RNA which imply that plant mitochondria have a eubacterial origin distinct from those of all other organisms (Yang *et al.* 1985, Gray *et al.* 1989). Ribosomes are found in all mitochondria and comprise part of the genetic system that expresses genes encoded on the equally ubiquitous mitochondrial DNA. This genetic system is distinct from that used to express nuclear and chloroplast genes, and is thought to be a relic of the original endosymbiont.

In all species studied mitochondrial DNA (mtDNA) apparently encodes a very similar set of less than 30 polypeptides and about 30 transfer and ribosomal RNA molecules (Hack and Leaver 1983, Leaver and Gray 1982, Lonsdale 1988). These contribute to either the genetic system or the oligomeric protein complexes in the inner mitochondrial membrane which are involved in electron transport (section 1.3.2). Two dimensional gel electrophoresis reveals about 300 polypeptides in mitochondria; most of these must therefore be encoded outside the mitochondria. In all known instances this is in nuclear DNA, and after synthesis in the cytosol the proteins are imported into the mitochondria. Some RNA molecules also are now thought to be imported (section 1.5.2). Thus mitochondria are far from autonomous; not one of the complexes or activities to which mitochondrial genes contribute can be completed in the absence of nuclear gene products. It is assumed that many of the genes now encoded by the nucleus were, during evolution, transferred from the genome of the endosymbiotic progenitor. Variation between organisms in their complement of mitochondrial genes is thought to reflect retention of slightly different members of a largely similar group. The reason for retention of this group of genes, less than 10 % of the total, and of the complex genetic system that is required to maintain express and regulate them, remains obscure (Jacobs and Lonsdale 1987).

Polypeptides synthesized in the cytosol and destined for the mitochondria

follow complex and variable import pathways. Import has been investigated in greatest detail in yeast and *Neurospora crassa*. Most proteins, exemplified by the β subunit of the F1-F0 ATP synthase (ATPB), are synthesised as precursors with N terminal extensions that target the remainder of the polypeptide to the mitochondria. The presequences of different mitochondrial proteins bear little primary sequence homology, though they possess abundant basic and hydroxylated residues, and few acidic ones (Allison and Schatz 1986). Perhaps their most important feature is that they generally fold to produce an amphiphilic α helix (von Hejne 1986, Eilers *et al.* 1988). Import can be effected after translation is complete, and this is thought to be the case *in vivo*. An electrostatic potential across the inner membrane is required to initiate import, and either ATP or GTP are necessary to maintain and complete it. Eilers*et al.* (1988) have evidence that the nucleotides are necessary to unfold or maintain the unfolded state of the precursor protein during import. Contact sites between the two membranes are thought to be the points through which the proteins pass (Nicholson and Neupert 1988). When in the matrix, the precursors are cleaved by a specific protease to release the presequence (Nicholson and Neupert 1988).

Proteins of the intermembrane space or outer surface of the inner membrane, such as cytochromes b1 and c respectively, are first imported into the matrix as above. Cleavage of the presequence during import reveals a second possibly ancestral signal sequence that redirects the protein back across the inner membrane, and this sequence is then also removed by a second protease in the intermembrane space (Hartl *et al.* 1987).

There appear to be several distinct import pathways. For example, neither cytochrome c nor the adenine nucleotide translocator (ANT), both located in the inner membrane, contain cleavable presequences. Apocytochrome c is apparently able to spontaneously insert into the outer membrane, from which its translocation and insertion into the inner membrane is dependent upon addition of haem. ANT initially binds to a proteinaceous receptor distinct from the ones used by ATPB and is then transferred to the contact sites. The N terminal third of the *S. cerevisiae* ANT protein, and the C terminal two thirds of the *N. crassa* protein apparently contain sufficient information for import (Adrian *et al.* 1986, Pfanner and Neupert 1987).

Little is known of the pathway for import of cytosolically synthesised proteins into plant mitochondria. ATPB from tobacco possesses a presequence which when fused to foreign proteins directs them into mitochondria (Boutry *et al.* 1987). Similarly, a presequence is present at the N terminus of a tobacco mitochondrial super-oxide dismutase which can be imported into yeast mitochondria where it is processed to give a product identical to that isolated from plants (Bowler *et al.* 1989). The import mechanisms for these proteins probably resemble those of the fungi. The ANT from maize (Baker and Leaver

1985) differs from its fungal counterparts in that it is apparently synthesised as a larger precursor and is processed to its mature form upon import (Purdue 1988). A processing activity has been identified in plant mitochondria (Purdue 1988, C. J. Sarah personal communication).

There is circumstantial evidence that proteins may also be transported from the mitochondrion; in mice inheritance of a cell surface antigen is maternal with its expression and allelic form dependent upon the presence and structure of the mitochondrial DNA respectively (Fischer-Lindhal 1985).

Characteristic Features of Plant Mitochondria.

Plant mitochondria differ from those of other organisms in several ways, as may be expected if their derivation is indeed distinct from the other organelles.

Plant mitochondria possess a number of unusual biochemical features (discussed in Douce 1985), though the mitochondrial genome is not known to contribute specifically to these. In addition to the usual rotenone sensitive NADH dehydrogenase activity of the inner membrane Complex I they possess three other such activities; one in the outer membrane that is unlinked to ATP production, and two in the inner membrane. Of the latter two, one is capable of directly oxidising cytoplasmic NADH, and the other is rotenone insensitive and capable of oxidising mitochondrial NADH (Moller 1986).

The activity of a cyanide resistant "alternative oxidase" has been detected in a number of species, and recently purified from the spadices of *Sauromatum guttatum* by Elthon and McIntosh (1987). A physiological role for the pathway is clear only in thermogenic organs such as these. Electron transport by-passes two sites of coupling to ATP synthesis, so the energy is dissipated as heat raising the temperature by as much as 15 ^{0}C and volatilising insect attractants, or providing frost resistance. Increased cyanide resistant respiration has been observed in aging potato tubers and in ripening fruit. It has also been shown that seed germination and early seedling growth is impaired by inhibitors of the alternative pathway; the significance of these observations is unclear (Day *et al.* 1983)

Photosynthesis fixes oxygen as well as carbon dioxide. The glycolate that eventually results from the oxygenase activity of ribulose bisphosphate carboxylase is respired by the photorespiratory pathway. This involves two mitochondrial enzymes, glycine decarboxylase and serine hydroxymethyl transferase, that are specifically synthesised in photosynthetic tissues. Another enzyme not generally found in mitochondria from organisms other than plants is NAD linked malic enzyme. It is responsible for decarboxylation of the malate that accumulates from fixation of atmospheric carbon dioxide in C4 and crassulacean

acid metabolic (CAM) plants. The carbon dioxide so released can be refixed via the normal photosynthetic pathway under conditions that reduce either photorespiration or water loss. In C4 plants this occurs in the bundle sheath cells that accumulate a high CO_2 and low O_2 concentration thereby reducing photorespiration; in CAM plants fixation of carbon as malate occurs in the dark, thus in the light CO_2 for photosynthesis is supplied from within, allowing the stomata to remain closed and reducing water loss (Ruess *et al.* 1988).

The molecular genetics of plant mitochondria will be discussed in detail in the following sections, but some of its characteristic features are noted below. Firstly, their genomes are the largest known, ranging up to 2,400 kb (Ward *et al.* 1981). They encode a unique complement of genes including one for the α subunit of the F1-F0 ATP synthase, (Hack and Leaver 1983, Isaac *et al.* 1985a) not found in any other mtDNA, and ones that potentially encode a reverse transcriptase and three protein subunits of the ribosome that are similarly unique (Schuster and Brennicke 1987a, Bland *et al.* 1986, Wahleitner and Wolstenholme 1988 and Gualberto *et al.* 1988). Plant mitochondrial ribosomes are amongst the largest described (Leaver and Gray 1982) and contain a 5 S rRNA not found in ribosomes from other mitochondria (Leaver and Harmey 1976).

The genetic codes used by animal, fungal and protistan mitochondria are different from each other and from the standard code. The one used by plant mitochondria is different from all other mitochondrial codes, and possibly also from the standard code (Fox and Leaver 1981).

Commercial interest in mitochondrial genetics was aroused by the discovery of cytoplasmic male sterility (CMS, reviewed in Leaver and Gray 1982, Laughnan and Gabay-Laughnan 1983). This trait, characterised by failure to shed functional pollen, is maternally inherited and has been observed in over 140 species. It probably results from lesions in the mitochondrial genetic system. As discussed in the following sections, CMS cytoplasms have altered mitochondrial DNA arrangements, altered transcription patterns and synthesise variant polypeptides. Mitochondrial genes show non-mendelian inheritance and, as mitochondria are generally excluded from pollen, mitochondrial DNA is inherited from the female parent alone.

Production of high yielding hybrid varieties in crop species that are normally able to self pollinate is greatly facilitated if one parent is male sterile thus ensuring that its progeny result from pollination by another individual. Fortunately for the plant breeder such male sterile cytoplasms can, in many cases, be restored to fertility by the presence of nuclear RF alleles. In the production of the hybrid, these alleles are transferred to the progeny from the male parent ensuring the fertility of the plants grown from the hybrid seed.

Three distinct forms of male sterility exist in maize; cms-C, cms-S, and cms-T. Each can be distinguished by the physiological process that is disrupted

during androgenesis, by the form of the mitochondrial DNA, but primarily by the RF loci required to restore fertility. All three have been used in hybrid seed production, most notably cmsT which was used in 85 % of all hybrid maize grown in the United States of America in 1970. The use of this cytoplasm was abruptly halted thereafter, following the devastation of the maize crop by the ascomycete *Helminthosporium maydis* race T which was virulent specifically on plants with the cmsT cytoplasm. This disaster stimulated much research into the causes of disease susceptibility and male sterility in general, and into methods for identifying and generating alternative sterile cytoplasms.

The only other phenotype known to be associated with a mitochondrial lesion in higher plants is Non Chromosomal Stripe in maize. This is characterised by pale and necrotic sectors in the leaves, and in extreme cases growth is severely retarded (Newton and Coe 1986). The trait occurs in allelic forms with distinguishable phenotypes. NCS2 mitochondria accumulate reduced amounts of a single polypeptide, and show altered transcription of a region of mtDNA that does not hybridise with any known plant mitochondrial gene (Feiler and Newton 1987).

1.2 Genetic Transformation.

The term transformation has several meanings, but its use in this sense has a long history. In 1928 it was discovered that an avirulent mutant of one strain of *Pneumococcus* could become virulent by treatment with an extract from killed cells of a different virulent strain. Virulence was associated with production of polysaccharides and strains could be distinguished by the type of polysaccharide they produced. The acquisition of virulence by the mutants was accompanied by synthesis of polysaccharides characteristic of the other, virulent, strain. Of particular importance was the observation that these newly acquired characteristics were inherited by future generations. This permanent heritable change in phenotype and by inference in genotype was termed transformation. With the elucidation of DNA as the Transforming Principle (Avery *et al.* 1944) and the genetic substance (Watson and Crick 1953a,b, Meselson and Stahl 1958), the term transformation has been applied to many instances where cells acquire new DNA molecules which then become inherited as part of the genetic material; acquisition of new DNA molecules through normal genetic exchange, hybridisation, or cell fusion, is usually considered to be distinct. In practice transformation is achieved either by a chemical or physical treatment that increases the efficiency with which cells take up purified DNA from the surrounding solution, or by including foreign DNA in an infective agent such as

a virus or bacterium that then delivers the new sequences to the genome of the host cell.

Despite its importance in initiating molecular genetics, transformation was of relatively limited practical significance until the advent of *in vitro* techniques for specifically manipulating nucleic acids. For prokaryotes and latterly eukaryotes these increasingly powerful techniques have provided a structural and mechanistic explanation for observations made during detailed genetic analyses.

The key to much of this progress has been the ability to isolate specific nucleic acids, to determine their structure and abundance, and to correlate changes in these parameters with particular physiological processes or defined mutant phenotypes; the isolation and analysis of such sequences from any organism is dependent upon their purification and amplification (cloning) that results when they are used to transform convenient organisms such as *E. coli*. Yet for developing functional explanations of gene action perhaps the most rewarding approach has been to introduce specific alterations into an isolated DNA sequence and to determine their effect upon its function in a suitable *in vitro* system, or preferably *in vivo* following transformation of the organism, from which the sequence was originally derived.

Replacing the coding sequence of a gene of interest with one that is easily assayed (a reporter gene) greatly facilitates analyses of the control regions that regulate gene expression in cells at different developmental stages, in different environments, or in different tissues of multicellular organisms. This approach is not confined to analysis of genes themselves, but can be extended to their products. Performing the reciprocal manipulation, the coding sequence of a gene can be expressed from control sequences with different temporal or spatial specificity and in cells that do not normally express the gene, allowing the role of the gene product to be investigated. Purification of large amounts of rare proteins for antibody preparation or structural analysis can be aided by expressing their genes at extremely high levels in *E. coli* after provision of the necessary regulatory sequences *in vitro*. The function of the structures so determined can be minutely dissected by inserting deliberate single base pair substitutions into their genes *in vitro*, so making specific amino acid substitutions in the proteins synthesised *in vivo* after transformation. A proper understanding of cancers or the biology of viruses and their associated lesions in prokaryotes and eukaryotes is not conceivable without many of the experimental approaches outlined above. Finally, via transformation with cloned sequences organisms may be given new genetic functions, some for their own benefit such as disease resistance, and some to facilitate production of substances for human benefit.

Already the mechanism of developmental regulation of one virus, bacteriophage λ, has been determined in atomic detail (Ptashne 1986), and fundamental signals controlling development in animals ranging from insects to

mammals (Gehring and Hiromi 1986) have been identified. Factor IX, a protein whose absence or malfunction causes haemophilia is now made by transgenic sheep and secreted into the milk from which it can be recovered (this and similar developments are discussed by Van Brunt 1988). Plants have been produced that are resistant to herbicides, viruses and herbivorous insects (Weising *et al.* 1988). Recent strategies to combat Acquired Immunodeficiency Syndrome rely on identification of the cellular receptor for the virus, isolation of its gene, fusion of this with part of an antibody gene to increase the half-life of the receptor in the blood, and the production of this recombinant protein from mammalian cells; all of these steps required transformation of either bacteria or mammalian cells (Capon *et al.* 1989).

The current interest in plant molecular genetics, as exemplified by the number of recent publications on the subject stems from the recently developed techniques for generating transformed plants (section 1.4). Transformation has also stimulated activity in established areas of plant molecular genetics; the work in many current papers involves gene isolation and could have been performed before transformation of plant cells was possible, but only now is there a chance of such work leading to a functional understanding of plant molecular genetics.

As it will become clear in the following section, the molecular genetics of plant mitochondria is confined to a description of nucleic acid structure, abundance and organisation in ever increasing detail. Progress towards a functional, mechanistic description is hindered by our inability to transform mitochondria with new or altered sequences or to generate informative mutations. The work described in this thesis is intended to provide that opportunity.

1.3 Molecular Genetics of Plant Mitochondria.

The preceding sections may be summarised as follows. Mitochondria possess genomes that contribute to their own genetic systems, which in turn are necessary to provide functions central to their vital metabolic role. Knowledge of both the activity and organisation of the genomes has accumulated, making it clear that an understanding of these is a prerequisite to a proper understanding of mitochondrial biology. Currently very little is known of the way in which the mitochondrial genetic system performs its crucial role. It is the elucidation of this that may be facilitated by transformation. This section reviews our current understanding of the system.

1.3.1 The Structure and Organisation of Plant Mitochondrial Genomes.

The mitochondrial genomes in higher plants range in size from 208 kb in *Brassica hirta* to 2,400 kb in *Cucumis melo* (Ward *et al.* 1981, Palmer and Herbon 1987). If all the Viridiplantae (Cavalier-Smith 1986) are considered, the range extends to the 16 kb genome of *Chlamydomonas reinhardtii* (Gray and Boer 1988). Plant mitochondrial genomes are both larger and more variable than those of any other group of organisms; in the Metazoa they range from 15 kb to 17 kb, and in the fungi from 19 kb to 176 kb (Dujon 1983, Clark-Walker *et al.* 1981, Hintz *et al.* 1985).

In addition to the main genome there are numerous reports of small linear or circular plasmid like molecules that appear to be independently maintained within the mitochondrion. Eighteen different circular plasmids have been reported from six plant species (Lonsdale *et al.* 1988). All but one are between 1.3 kb and 2.3 kb, and many are transcribed though no coding function has been assigned to them.

Linear plasmids have been found in four species. They range from 2.1 kb to 11.3 kb, are double stranded, with terminal inverted repeat (TIR) sequences and proteins attached to their 5' termini. The best studied of these are the S1 and S2 plasmids that characterise the CMS-S maize cytoplasms. They are 6.4 kb and 5.4 kb respectively (Pring *et al.* 1977), with TIRs of 208 bp. Both plasmids encode open reading frames (ORFs, Paillard *et al.* 1985) that are transcribed (Traynor and Levings 1986). ORF 1 of S2 and ORF 3 of S1 are known to be translated (Manson *et al.* 1986, Zabala *et al.* 1987 respectively), and it has been proposed that the former encodes an RNA polymerase (Kuzmin *et al.* 1988), and the latter a DNA polymerase (Kuzmin and Levchenko 1987). In contrast to other plasmids, S1 and S2 are homologous to parts of the main mitochondrial genome (Koncz *et al.* 1981, Houchins *et al.* 1986). In the genomes of normal, fertile (N-type) cytoplasms over 90 % of their sequence is represented, though frequently mutated, adjacent to one or other copy of a 5.27 kb repeated sequence. In the genomes of CMS-S cytoplasms, sequence similarity with the S-plasmids has been reduced to two regions homologous to the terminal 186 bp of the TIRs. It appears that in S-type cytoplasms, these plasmid homologous sequences have been transposed from their position next to the 5.27 kb repeat to two other locations, one of which is adjacent to the COXI gene. The genome may recombine with each end of the S plasmids at these points, to generate a variety of integrated forms (Schardl *et al.* 1984). These recombination events occur close to the gene for subunit 1 of cytochrome c oxidase, and this is relevant to my work (Chapter 3).

The 2.3 kb linear plasmid in maize is unique in that it encodes what is

thought to be the only gene in the mitochondria for $tRNA^{trp}$ (Bedinger *et al.* 1987, Marechal *et al.* 1987). No other plasmid encoded function has been suggested to contribute to the normal functioning of the mitochondrion.

Certain cytoplasms of maize contain single and double stranded RNA molecules that appear to replicate without DNA and independently of the main mitochondrial genome (Sisco *et al.* 1984, Finnegan and Brown 1986).

The variable nature and distribution of these plasmids is not surprising given that their contribution to mitochondrial function is doubtful. What was unexpected was the discovery that plant mitochondrial genomes encoding the same basic set of proteins and RNAs differ so widely in size and organisation, but particularly in size. This variation cannot be explained by the presence of differing amounts of highly repetitive sequence; the reassociation kinetics of mitochondrial DNA from four species of the Cucurbitaceae showed that less than 10 % of their genomes are highly repetitive despite a seven fold difference in size (Ward *et al.* 1981). The significance and origin of the structural variation remain to be fully explained, however analysis of genome structure, described below, has revealed several contributory factors.

The mitochondrial DNA of nine plant species has been mapped by ordering overlapping restriction fragments (reviewed in Lonsdale et al. 1988), and can be represented as a single circular molecule like those of Metazoa and most fungi. In contrast, electron microscopic examination of purified mitochondrial DNA reveals predominantly linear molecules (Wolstenholme and Gross 1968, Synenki *et al.* 1978, Manna *et al.* 1985) with a small proportion of circular molecules of up to 100 kb. Dale *et al.* (1981) were able to isolate greater than 20 % of mitochondrial DNA as circular molecules from tissue cultures of three species. Shearing of such molecules has been deemed responsible for generating the linear species though it is possible that the genome exists as a set of circularly permuted linear molecules. In fact, following recombination with the linear S and R plasmids, some regions of the RU and S maize genomes have been shown to be linear (Schardl *et al.* 1984, Lonsdale *et al.* 1988). The genomes of other organisms including *Tetrahymena thermophila*, *Chlamydomonas reinhardtii* and the yeast species *Candida rhagii* are also linear (Morin and Cech 1986, Gray and Boer 1988, and Kovac *et al.* 1984).

Even though those plant mitochondrial genomes analysed to date can be represented as a single 'master' circle, it is now thought that in most cases the genome is multipartite, comprising two or more subgenomic circles. Evidence for this has come from restriction mapping and electron microscopy. The subgenomic molecules appear to arise from recombination between repeated sequences of up to 14 kb distributed throughout the genome; for example the WF9-N maize mitochondrial genome is 570 kb, contains six repeats of between 1 and 14 kb, of which one is inverted and five are direct, and one of these includes the entire coding sequence of the gene for ATPA (Lonsdale *et al.* 1988, Isaac *et al.*

1985a). The mitochondrial genome of only *Brassica hirta* , which is the smallest known and uniquely lacks such repeats, appears to exist as a single circular molecule (Palmer and Herbon 1987). These repeats contribute to the complexity and variability of the mitochondrial genomes.

Recombination is prevalent in plant mitochondria appearing to greatly influence the organisation and evolution of the genome (Lonsdale *et al.* 1988). It seems that it is the sequence duplication itself, and not specific sequences within it that are important in recombination implying that general homologous recombination is responsible (Lonsdale *et al.* 1988). The repeats involved above are larger than 1 kb, but Small *et al.* (1987) have found some subgenomic molecules to be present at very low copy number, and these are probably products of recombination between much smaller repeats. Amplification of such molecules has been proposed to account for some features of maize genome evolution (Small *et al.* 1989). Other small repeats of between 55 bp and 677 bp have been found (compiled in Lonsdale *et al.* 1988), and though the prevalence of such sequences is unknown, they must also contribute to the variability in size and structure of the genome.

In *Oenothera*, the products of recombination between repeats of less than 15 bp have been detected, and a site specific recombination mechanism has been invoked (Manna and Brennicke 1986, Schuster and Brennicke 1986, Wissinger *et al.* 1988). In one such case involving the 26S rRNA gene (*rrn26*), not all of the four products expected from a reciprocal recombination event were detected, suggesting that rare homologous recombination and amplification of some of its products may be involved as postulated by Small *et al.* (1989). These and other events occur in the coding or transcribed spacer regions of several protein encoding genes and rRNA genes, thereby generating inactive pseudogenes. Again this type of repeated sequence adds to the diversity in organisation of gene expression.

Intragenic recombination seems also to have been responsible for the assembly of novel chimaeric open reading frames that encode polypeptides implicated in cytoplasmic male sterility. For example, CMS-T maize cytoplasms contain an open reading frame called T-*urf13* which encodes a 13 kd polypeptide; the sequence in this region of the genome was generated by at least six recombination events involving a tRNA gene of chloroplast origin, 5' sequence flanking *atp6* and 3' coding and flanking sequence from *rrn26* (Dewey *et al.* 1986, genetic loci are defined in section 1.3.2). Functional copies of the two rearranged genes exist elsewhere in the genome. In CMS-C, the promoter and N terminal coding region of *atp6* replace those of *coxII* to give a new chimaeric open reading frame (Levings and Dewey 1988). The remainder of *atp6* is fused to sequences derived from *atp9* and the chloroplast genome to create a second new reading frame. Remarkably, no unmodified forms of *coxII* or *atp6* were found in the

CMS-C genome, suggesting that their modified N termini are largely tolerated. In this context it may be of relevance that in yeast, assembly of COXII into complex IV is accompanied by processing of its N terminus (Pratje *et al.* 1983). Sequence analysis has revealed extensive variation in the N termini of *atp6* in several plant species (Bland *et al.* 1987, Schuster and Brennicke 1987d, Grabau *et al.* 1988) which is either tolerated or removed from the protein. These observations suggest that the gene modifications in CMS-C might be less disruptive than they appear.

Finally several sequences derived from the chloroplast and nuclear genomes have been detected in mitochondrial DNA from a number of plants; these observations are discussed in detail later in this chapter.

The size of plant mitochondrial genomes is thus determined in part by the presence of varying numbers of differently sized repeated sequences, some of which have no known coding function (Houchins *et al* 1986), some are pseudocopies of active genes, and some contain genes that are apparently fully active (Isaac *et al.* 1985a, Rothenberg and Hanson 1987b). It is probable that recombination between these repeats on the putative master circle and the subgenomic circles is responsible for the complex genome structures that are observed.

1.3.2 Coding Capacity of Plant Mitochondrial Genomes.

Plant mitochondrial genomes contain the same basic set of genes identified in those of animals and fungi. In addition they contain others that have been shown to be located in the nuclei of these organisms.

Genes isolated from plant mitochondrial DNA include *rrn26* , *rrn18* , and *rrn5* (Lonsdale 1988), encoding respectively the large and small rRNA molecules common to all ribosomes, and a 5 S rRNA that is present in mitochondrial ribosomes from only plants (Leaver and Harmey 1976). The genes for 14 different tRNAs that accept 11 different amino acids and include an initiation specific $tRNA^{f\text{-}met}$ have also been isolated (compiled in Lonsdale 1988). Most mitochondrially encoded tRNAs are very similar to those in chloroplasts, however four $tRNAs^{leu}$ isolated from *Phaseolus vulgaris* mitochondria are identical to their cytosolic counterparts except for post-transcriptional modifications typical of mitochondrial tRNAs. Because they hybridise to nuclear but not mitochondrial DNA they may be imported from the cytosol (Marechal-Drouard *et al.* 1988). In fungi and mammals all mitochondrial tRNAs are encoded within the organelle, however the genomes of *C. reinhardtii* and *T. thermophila* do not encode all the necessary tRNAs, the remainder presumably

being imported (Suyama 1986, Gray and Boer 1988).

Sterile isolated plant mitochondria, in the presence of amino acids and an energy source, are able to synthesise protein (Forde *et al.* 1978, 1979). By supplying radioactive amino acids followed by analysis of the labelled products by two dimensional electrophoresis, Hack and Leaver (1983) concluded that between 30 and 50 polypeptides were synthesised within the mitochondria and presumably encoded in mtDNA. This is significantly more than mammalian and fungal mitochondria which encode 13 and about 15 polypeptides respectively. However it is unlikely that every labelled product was derived from a different gene, because some probably arose by charge modification of common polypeptides. A more reasonable estimate is 20 to 30 genes; 24 transcription units have been mapped on the *Brassica campestris* mitochondrial genome (Makaroff and Palmer 1987). Four species of the Cucurbitaceae all synthesise very similar sets of polypeptides despite their genomes ranging from 330 kb to 2,400 kb (Stern and Newton 1985); the largest of these is half the size of the *E. coli* chromosome which is thought to encode two to three thousand genes (Glass 1982). Thus the apparent coding capacity of plant mitochondrial genomes does not explain their large and variable size.

Transcribed reading frames encoding 13 different polypeptides have been identified in plant mitochondrial DNA, excluding those generated in CMS cytoplasms (compiled in Lonsdale 1988). Functions have been assigned to all of these primarily by comparing the amino acid sequence of their predicted products with that of known proteins. These include *cob* , *coxI* , *coxII* , *coxIII* , and *atp6* which encode apocytochrome b of the bc_1 complex, subunits 1 , 2, and 3 of the cytochrome c oxidase complex, and subunit 6 of the F1-F0 ATP synthase complex respectively. These genes are found in the mitochondria of mammals and fungi. In contrast, genes encoding subunits 9 and α of the F1-F0 ATP synthase have also been isolated, though they are nuclear in mammals and some fungi. Their products have been detected by DCCD and antibody binding (Hack and Leaver 1983, 1984, Boutry *et al.* 1983). Genes encoding subunits 3 and 5 of the NADH ubiquinone oxidoreductase complex (*nad3* and*5* respectively) have been sequenced, and a transcribed reading frame that is homologous to an internal portion of subunit 1 (*nad1*) has been found in several species (Chapter 5). It is present but not transcribed in *B. campestris* (Makaroff and Palmer 1987), so its coding function is unclear.

The reading frames *rps12* , *rps13* and *rps14* encode proteins homologous respectively to the small ribosomal subunit proteins 12, 13 and 14 of *E. coli.* Homology to *rps13* cannot be detected in pea, bean or soybean mitochondrial DNA, and though present and highly conserved in wheat, no transcripts could be detected (Bland *et al.* 1986, Bonen 1987). Similarly, there is homology to *rps14* in broad bean and soybean but not in maize (Wahleitner and Wolstenholme 1988).

In *S. cerevisiae* and *N. crassa* the small ribosomal subunit proteins var1 and S5 respectively are mitochondrial gene products (Butow *et al.* 1985, Lambowitz *et al.* 1979), though their sizes suggest that they are unlikely to be equivalent to any of the plant mitochondrial *rps* loci (Bland*et al.* 1986, Wahleitner and Wolstenholme 1988, Gualberto *et al.* 1988).

The apparent similarity between the genetic complement of mtDNA from plants and other organisms may have been over emphasised as genes occurring in other mtDNAs are sought actively in plant genomes whereas the remainder are discovered largely by chance; as discussed above several unique to plant mitochondria have been discovered nonetheless. How many more exist, and what contribution they make to genome size variation remain to be established.

Some mitochondrial genes are split by introns. These are sequences that interrupt the coding sequence of the gene and its primary transcript, and are subsequently removed to generate a contiguous coding sequence in the mature messenger RNA. They are present in *coxII* in monocots, but in only some of the dicots analysed to date, and in *nad1* and *nad5* .

Genetic loci have been mapped on several genomes, but their arrangement is not conserved (Dawson *et al.* 1986a, Makaroff and Palmer 1987). The only conserved arrangements are the 18 S and 5 S rRNA genes that are cotranscribed as a single precursor, and the *rps13* and *nadI* homologous sequences that are generally also cotranscribed. It is doubtful whether cotranscription is the sole reason for linkage because in the two instances mentioned previously in which only one of the latter pair of genes is transcribed their linkage is maintained. This variable gene order reflects the high rate of rearrangement of coding and non-coding sequences in plant mtDNA. Between 3 and 14 inversions and up to eight deletions, duplications or insertions are required to account for the major structural differences between any pair of mtDNAs from six species of *Brassica* and *Raphanus* (Palmer and Herbon 1988). The genome size of these species, which ranges from 208 kb to 242 kb, has been relatively unaffected by these events. Palmer and Herbon (1988) note that this poor conservation of genome structure is in stark contrast to vertebrate mtDNA structure in which gene order is essentially invariant, and to the structure of the chloroplast DNA (ctDNA) of land plants that rarely undergo internal rearrangement. A further contrast is drawn between the relative base substitution rates which in these plant mtDNAs are four times lower than in land plant ctDNA and 100 times lower than in vertebrate mtDNA. Palmer and Herbon (1988) conclude that 'the *Brassica* mitochondrial genome may be viewed as a collection of unchanging sequences whose relative arrangement is extremely fluid'. This conservation of sequence may underlie the similarity in bouyant densities amongst higher plant mtDNAs (Leaver 1975).

Thus an increasingly detailed description of the structure and organisation of plant mitochondrial genomes is emerging. From these investigations, the basic

unifying features and much of the potential variability of plant mtDNAs have probably been described; however, it is proving difficult to progress from this to a mechanistic understanding of how plant mitochondrial genomes function. The roles of some open reading frames, the details of the recombination process, and the influence of these on normal mitochondrial function and inheritance, are open to speculation, but not experimental verification. The inability to progress from detailed description to mechanistic understanding of a process is particularly clear when gene expression and its regulation is addressed. As yet we have only a tentative understanding, based largely on circumstantial evidence, of even the basic requirements and mechanisms of replication, transcription, and translation of mitochondrial nucleic acids. This is briefly summarised below.

1.3.3 Expression of Plant Mitochondrial Genes.

The basic processes involved in gene expression are transcription of the gene to generate a complimentary RNA molecule, followed usually by translation of the RNA to synthesise the protein it encodes. Transcription is performed by RNA polymerase which recognises specific sequences in the genome, called promoters, that determine the points at which transcription initiates. Transcription generates ribosomal, transfer and messenger RNAs (rRNA, tRNA, and mRNA). The rRNAs are structural components of ribosomes. During protein synthesis, tRNAs deliver specific amino acids to the ribosomes which, in combination with a number of other factors, catalyse the polymerisation of these amino acids in the order encoded in the mRNA.

Analysis of mitochondrial genome transcription in other organisms showed it to be achieved in two different ways. In vertebrate mitochondria, the genes are tightly linked and transcription initiates at two divergent promoters to generate large precursor molecules which are cleaved to produce the individual structural and messenger RNA species (Foran *et al.* 1988). In yeast, the genome contains relatively large intergenic regions in which transcription initiates. This occurs at variable distances upstream from the coding sequence of mRNAs and from the processing sites at which transfer and ribosomal RNA precursors are cleaved to generate the mature molecules; for example 540 nucleotides (nt) in the COXI mRNA, and 135, 75, and 0 nt respectively upstream of the mature $tRNA_1^{Thr}$, 15S rRNA and 21S rRNA (Osinga and Tabak 1982, Osinga *et al.* 1984b). Genes are either transcribed singly or in groups usually of less than five (Mueller and Getz 1986a).

The large genome size and occurrence of extensive sequences that do not have a clear coding function suggested that plant mitochondrial gene expression

would more closely resemble that of yeast than vertebrates. Early studies on four species in the *Cucurbitaceae* , whose mitochondrial genomes varied in size by seven fold, showed that only a fraction of genomic restriction endonuclease fragments from even the smallest genome hybridise to total mtRNA, (Stern and Newton 1985). This hybridisation in all but the largest genome was to sequences common in the four species, encompassing about 50% of the smallest and 30% of the largest genome. In a more systematic study, Makaroff and Palmer (1987) probed Northern blots with labelled restriction endonuclease fragments encompassing the entire mitochondrial genome of *Brassica campestris*, and identified 24 distinct transcription units. These covered 61 kb, 30% of the total genome; however numerous less abundant overlapping transcripts were also found, and not all were associated with the abundantly transcribed fragments. This form of analysis did not exclude the possibility of there being low abundance transcripts from the apparently silent regions, or that these regions were expressed in tissues or at developmental stages that were not analysed; Bonen (1987), Brown *et al.* (1984) and van den Boogaart *et al.* (1982) have proposed the latter alternatives as explanations of the apparent absence of transcription of *rps13* in wheat and *atp9* in *Aspergillus* and *Neurospora* respectively.

Carlson *et al.* (1986) found that radioactive RNA synthesised by isolated maize mitochondria hybridised to all mitochondrial restriction endonuclease fragments, though not in equal measure. These results, which have not been extended or confirmed, suggested that plant mtDNA may be transcribed in a fashion similar to that of vertebrates.

Northern Blot hybridisation analysis with an increasing number of cloned mitochondrial genes has revealed that several generate multiple transcripts some of which extend several thousand nucleotides upstream or downstream of the coding sequence. However, in agreement with the results of Makaroff and Palmer (1987), at sufficient but variable distances from coding sequences, transcripts were no longer detectable (for example Isaac *et al.* 1985b, Wissinger *et al.* 1988). The sequences around transcript 5' termini were found to be variable, but often fell into finite categories (Chapter 3). Conserved sequences around transcript 5' termini in yeast and vertebrate mtDNA have been shown to constitute at least part of the promoter sequence (discussed in detail in Chapter 3), and those plant mtDNA sequences which were similarly conserved were proposed by analogy to be potential promoters (for example Isaac *et al.* 1985b, Rothenberg and Hanson 1987, discussed in Chapter 3). Conserved stem loop structures near the 3' termini of several plant mitochondrial transcripts have been proposed to constitute termination signals (Schuster *et al.* 1986, discussed in Chapter 5). These results prompted the following view of plant mitochondrial gene expression: as in yeast, transcription in higher plant mitochondria probably initiates and terminates at several specific points in the genome; these points lie at varying distances from

the coding regions of several protein and structural RNA genes; cotranscription of genes is probably not the norm, though it is known for some genes (for example Bland *et al.* 1986, Wissinger *et al.* 1988); several promoters may exist for one gene, and in many cases, primary transcripts may subsequently be processed to generate some of the final messenger, transfer and ribosomal RNA species.

However, recent studies to distinguish primary transcripts from processed products (discussed in Chapter 3) suggest that almost all transcripts may arise by genuine initiation of transcription, and that the DNA sequences which constitute the promoter may be more variable and less obvious than previously supposed (Mulligan *et al.* 1988a,b). It has been proposed that mitochondrial RNA polymerase may interact with DNA non-specifically to initiate transcription, or that the specific sequences recognized as promoters may be dispersed, or lie at a distance from the initiation site.

Further details of the transcription and translation processes are discussed in Chapter 3 which addresses the design of chimaeric mitochondrial genes. The rest of this section will be concerned with evidence for control and regulation of gene expression in plant mitochondria.

1.3.4 Control of Mitochondrial Gene Expression.

The quantity of a gene product that accumulates can be affected not only by its stability, but also by the rates of transcription of its gene, maturation and degradation of the transcripts, and, if the product is a protein, translation of the mRNA. Products of different genes are accumulated in different amounts, for example the structural ribosomal RNAs are more abundant than any single message, and in the F1-F0 ATP synthase, three molecules of ATP9 are required for each of ATPA; these requirements can be met in part by differential efficiency of gene expression. This modulation of gene expression is distinct from spatial or temporal regulation and will be discussed first.

In yeast, the steady state levels of messenger, transfer and ribosomal RNAs were found to differ by up to 50 fold (Mueller and Getz 1986b). Furthermore, Mueller and Getz (1986a) used a solution hybridisation system to assay incorporation of pulses of radioactive label into specific transcripts during exponential growth of a culture. They concluded that the relative steady state levels of each transcript were proportional largely to the promoter strength and the rate of elongation of the transcript. The results of these experiments were in agreement with the relative strengths of five tRNA promoters determined by *in vitro* transcription of cloned sequences using partially purified mtRNA polymerase (Wettstein-Edwards *et al.* 1986). Promoter strength was found to differ

by 20 fold, and correlated with the identity of the two nucleotides immediately flanking a conserved nonanucleotide core sequence (Mueller and Getz 1986a, Chapter 3). These observations show that control of the rate of transcription is an important factor in determining the levels to which different transcripts accumulate, though transcript turnover through degradation has a contributory role (Mueller and Getz 1986b).

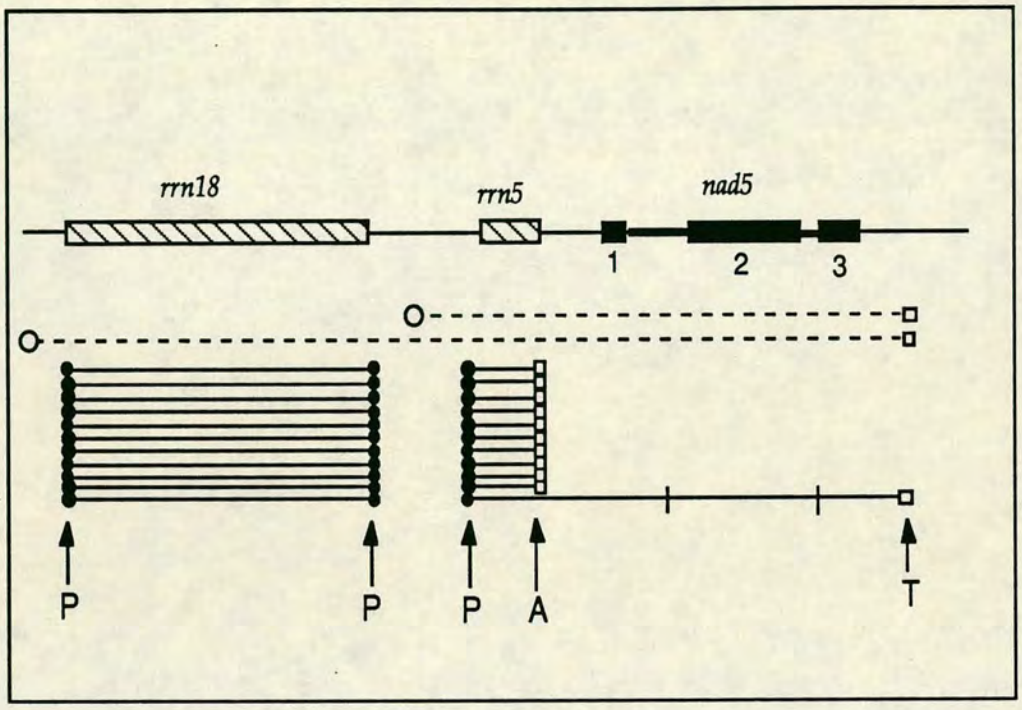
Some yeast genes are cotranscribed. In four different polycistronic transcription units, the rate of transcription of genes in the 3' portion was found to be less than those in the 5' portion. Such 'attenuation' was most marked downstream of the genes for $tRNA^{fMet}$ and $tRNA^{Glu}$ where transcription was reduced 17 fold (Mueller and Getz 1986a). Attenuation has also been described in mammalian mitochondria where all genes are transcribed from one of two divergent promoters. Transcription from the heavy strand promoter first encompasses the genes for $tRNA^{Phe}$,12 S rRNA, $tRNA^{Val}$ and 16 S rRNA, which all accumulate to high levels; transcription then continues to include almost all the remaining genes, though they accumulate to lower steady state levels (Clayton 1984). Potentially, such attenuation could be caused by slower polymerisation of the 3' region of the transcript, termination of most transcripts downstream of the abundantly transcribed region, or differential stability of different portions of the transcript. Mueller and Getz (1986a) concluded that differential stability alone was inadequate to explain attenuation, and of the remaining possibilities leaky termination seems more plausible.

Expression of *nad5* in *Oenothera* is the only documented instance of attenuation in normal plant mitochondrial gene expression (Wissinger *et al.* 1988). This gene is located downstream of the 5 S rRNA gene being cotranscribed with it and probably the 18 S rRNA gene. All transcripts homologous to *nad5* also contain the 5 S rRNA sequence in the 5' untranslated leader, and almost all terminate at the 5' end of the mature rRNA sequence; however, the mature 5 S rRNA accumulates in excess of the *nad5* mRNA (Figure 1.1). Efficient and leaky terminators, of a type that may be responsible for generating the 3' ends of the *nad5* and rRNA transcripts respectively, are thought to occur in plant mitochondria, their presence having been postulated downstream of two ATP9 genes in *Petunia* (Rothenberg and Hanson 1987b).

Expression of protein coding genes in yeast mitochondria is also influenced by a complex post-transcriptional control system. For example Ben Asher *et al.* (1989) have isolated three nuclear genes, NAM1, NAM7, and NAM8, that appear to be involved in pre-mRNA splicing, and directly in translation of mRNAs. An unexpectedly large proportion of respiratory deficient nuclear mutants are deficient in expression of only a single mitochondrial gene (Michaelis *et al.* 1982). These mutations seem to affect translation of specific messenger RNAs, though transcription appears normal. The implication is that for each mitochondrial gene

Figure 1.1

Organization and Transcription of *nad5* in *Oenothera* mtDNA.



The three exons of *nad5* are located downstream of the 18 and 5 S rRNA genes and are represented by the black boxes. Heavy lines between the boxes represent introns. Transcripts for *nad5* originate upstream of the *rrn18* and perhaps also in the spacer region between the two rRNA genes; these precursor transcripts are shown as dotted lines; their 5' ends arise from initiation of transcription (open circles), and their 3' ends from termination (T) beyond *nad5* (open squares). The solid lines below the precursors represent the mature, accumulated transcripts for each gene. Wissinger *et al.* (1988) have proposed that most primary transcripts terminate (A, open squares) near the 3' end of the 5 S rRNA sequence, the remaining rRNA termini being generated by processing (P) of the primary transcripts (black circles). A fraction of the the primary transcripts however continue past the putative terminator at the 3' end of *rrn5* and extend through to the terminator 3' of *nad5*. The 5' terminus of these transcripts could simply be generated by the same processing event that generates the 5' end of the mature 5 S rRNA.

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there is a specific set of nuclear functions that are responsible for translation of its mRNA; the products of such genes presumably contribute to controlling the quantity of each mitochondrial protein that is synthesised. For example, translation of *cob* mRNA requires at least two nuclear genes CBS1 and CBS2, which are thought to act on the 5' untranslated leader (Rödel 1986). Similarly, translation of *coxIII* mRNA is dependent upon three nuclear loci PET494, PET54 and PET55. The products of the first two loci are proteins that are known to be directed to the mitochondrion and probably act directly upon the 5' portion of the *coxIII* mRNA; translation of a rearranged form of *cob* that has its untranslated leader replaced by all but the 3' third of the *coxIII* leader becomes dependent upon these nuclear loci (Fox *et al.* 1988).

Interestingly, the maize nuclear gene RF1 involved in restoration of fertility to plants with the cmsT cytoplasm seems to act by altering the processing of the *turf13* transcripts by impairing their translation (Kennel and Pring 1989). The RF1 locus is likely to have a similar, role in expression of a second mitochondrial gene in all maize cytoplasms, and may be analogous to the PET or NAM loci of yeast.

Control of gene expression usually involves more than simply ensuring that genes are expressed at the correct levels relative to each other. In response to various stimuli the rate of synthesis of all gene products may be modulated, or the rate at which some products are synthesised may be altered relative to others. In fact in most cellular systems, these forms of genuine regulation of gene expression are probably of paramount importance during development or in response to changes in the environment.

For example in yeast the synthesis of mitochondrial gene-products encoded in the nucleus and mitochondrion is repressed if glucose is present in the medium; this is termed catabolite repression. Expression of the nuclear gene for iso-1-cytochrome c, *cyc1* , is influenced by the binding of at least four protein factors HAP1, 2 and 3, and RC2 to two sites upstream of the point where transcription starts (Pfeifer *et al.* 1987, Olesen *et al.* 1987). These proteins interact to alter transcription of *cyc1* , and one or more is involved in regulating transcription of genes for iso-2-cytochrome c, cytochrome b_2, cytochrome c oxidase subunit IV, and d-amino levinulinate synthase. Mueller and Getz (1986b) showed that under catabolite repression, the amounts of most ribosomal, transfer, and messenger RNAs in the mitochondrion decrease by about five fold, suggesting a general reduction in transcription. However, under these conditions, Fox *et al.* (1988) report reduced expression of *coxIII* as expected, but found that PET494 expression was also reduced. Combined with evidence that some mitochondrial mRNA species are unstable if they are not being translated it was proposed that transcription may be constitutive, and that mRNA accumulates to a lower extent as a result of catabolite repression reducing the synthesis of nuclear factors controlling its translation.

Not all regulation of mitochondrial gene expression occurs in this fashion. Rabbit striated muscle from different sources, and after different degrees of stimulation, varies in its capacity for oxidative phosphorylation. Williams (1986) and Williams *et al.* (1986) have shown that this capacity is directly proportional to the cellular levels of mitochondrial DNA, of mitochondrial ribosomal RNA and of mRNA for *cob* . Thus regulation may be effected simply by changes in gene copy number relative to nuclear DNA, though the capacity for gene specific regulation is clearly limited.

A number of developmental changes in higher plants are accompanied by alteration in the metabolic activity of mitochondria. This is perhaps to be expected as meristematic activity, early cellular growth and differentiation, and organogenesis are dependent upon regulated provision of energy and metabolic intermediates by mitochondria, which in turn must be able to oxidise different respiratory substrates in accordance with the changing metabolic status and physiological functions of differentiating cells. Examples include flowering, fruit ripening (Grierson 1986), early seedling development (Dixon *et al.* 1980), and the uncoupling of respiration in thermogenic tissues (Douce 1985). There are relatively few studies of altered gene expression accompanying such changes. Newton and Walbot (1985) showed there to be qualitative and quantitative differences in the proteins synthesised by maize mitochondria isolated from different tissues, and Forde *et al.* (1979) observed alterations in the proteins synthesised by mitochondria isolated from Jerusalem artichoke tubers after wounding. Topping (1987) showed transcription of mitochondrial genes to vary in successive sections along 7 day old wheat leaves. Similar changes were observed for the nuclear gene encoding the mitochondrial adenine nucleotide translocator.

Transcripts of the *pcf* gene that probably is responsible for male sterility in *Petunia* show a four to five fold increase in their steady state levels specifically in anthers; a second gene encoding *atp9*, similar to the one from which *pcf* is derived and whose upstream sequence is identical to that of *pcf* for at least 8 kb , shows no such increase (Young and Hanson 1987). Interestingly, the nuclear restorer gene does not affect transcript levels, suggesting that it acts post-transcriptionally as in the cases discussed above.

Bowler *et al.* (1989) report that infection of tobacco with the pathogen *Pseudomonas syrigae* or culture of various tissues in high concentrations of sucrose leads to increased levels of cytochrome c oxidase activity, requiring both mitochondrial and nuclear functions. Expression of a nuclear gene for a mitochondrial superoxide dismutase was similarly increased. Lastly, there appears to be diurnal fluctuation in the expression of the nuclear gene encoding the β subunit of the F1 ATP synthase in tomato fruits (Piechulla and Gruissem 1987).

These preliminary results suggest that there may be in plant cells, as in yeast, a series of interactions between sequences in mitochondrial nucleic acids and

regulatory factors from either the mitochondrion or the nucleus that combine to determine the spatial and temporal expression of mitochondrial genes. If transformation of plant mitochondria was possible, such interactions, once properly established, may potentially be analysed by isolation of the relevant mtDNA sequences which could then be modified *in vitro* and used to transform plant mitochondria. The information that may be obtained from this sort of analysis is likely to be of value if, in future, transformation is to be used to manipulate plant mitochondria through alteration of their genomes.

1.4 Transformation of Plant Cells.

Modification of plant genomes has been possible for thousands of years by conventional plant breeding, by inter specific hybridisation, and latterly by *in vitro* culture and cell fusion techniques (reviewed in Galun and Aviv 1986). These techniques can be extremely time consuming, frequently non-specific, and applicable to limited ranges of species and phenotypes. The current ability to transfer into plant cells a defined DNA sequence of any origin such that it creates a new and inherited genetic locus is the culmination of research during the last decade. This process is known as transformation. Of particular importance is the ability to transform plants with DNA molecules that have been subjected to the powerful range of *in vitro* manipulations that are currently available. As explained in section 1.2 this provides a unique opportunity to transfer new functions into crop plants and to investigate and modify existing ones, for example those that control the timing and level of gene expression.

Investigation and then exploitation of the DNA transfer system of the soil borne bacterium *Agrobacterium tumefaciens* initiated the development of the current range of plant transformation techniques. Experimental transfer of a novel foreign DNA sequence into plant cells, the transposable element Tn7 of *E. coli* , was first achieved in 1980 (Hernalsteens *et al.* 1980). This was followed by the successful transfer and phenotypic expression of a synthetic chimaeric gene that was designed to synthesise bacterial neomycin phosphotransferase in plant cells, thereby bestowing upon them resistance to the antibiotic kanamycin (Herrera-Estrella *et al.* 1983a,b). A recent compilation (Weising *et al.* 1988) reveals that in the first five years since this experiment over 50 different genes have been transferred into more than 30 monocot and dicot species using twelve distinct transfer techniques. *Nicotiana tabacum* has been most frequently used as a recipient for foreign DNA because the tissue culture and plant regeneration steps that are usually involved in the transformation process can be performed easily with this species. As an understanding of *A. tumefaciens* has been central to the

progress of plant transformation, a brief summary of its biology and exploitation will be given.

Most dicots, gymnosperms and some monocots are susceptible to Crown Gall disease which is characterised by tumorous growths, "crown galls", occurring at wound sites (Weising *et al.* 1988). Such tumours can grow in culture without being provided with auxin or cytokinin, the plant growth regulators that are required for culture of normal tissues. The causative agent of the disease is *A. tumefaciens*, a gram negative soil bacterium of the Rhizobiaceae (Zambryski *et al.* 1983a). Interest in the disease increased with the discovery that the tumour cells continued to grow indefinitely even when permanently freed from all the inciting bacteria (Braun and White 1943, Braun 1953). This implied that the tumorous growth resulted from a permanent, heritable alteration to the cells themselves, perhaps by some modification of their genetic material. A clue to the nature of this modification came with the discovery that a 20 kb portion of bacterial DNA, the T-DNA, became integrated into the nuclear genomes of crown gall cells (Chilton *et al.* 1980, Wilmitzer *et al.* 1980). Virulent strains of *A. tumefaciens* harbour large plasmids, about 200 kb, called Ti for tumour inducing (Van Larebeke *et al.* 1974,1975) from which the T-DNA originates. A comprehensive genetic analysis of the bacterium and its plasmid has revealed the mechanism of tumour induction (reviewed in Zambryski *et al.* 1983b).

Several chromosomal loci and a set of virulence (*vir*) functions on the plasmid result in plant cell recognition and attachment as well as excision transfer and perhaps integration of the T-DNA into the plant genome. The T-DNA region of the plasmid is defined by imperfectly repeated sequences of 25 bp that form its right and left border, and is physically separate from the *vir* region of the plasmid. The *virD* locus encodes a site specific endonuclease that cleaves the right border. A single stranded copy of the T-DNA is generated from this point and is attached, probably covalently, by its 5' end to the product of the *virD* 2 open reading frame (Stachel *et al.* 1986, Ward and Barnes 1988). How this structure is transported into the plant cell and integrated into the genome is not known, however it does seem to be a modified form of bacterial conjugation (Buchanan-Wollaston *et al.*. 1987, Lichtenstein 1987). Frequently more than one copy of the T-DNA becomes integrated either as direct or indirect repeats, and occasionally it undergoes structural modification (Lemers *et al.* 1980, Tomashow 1980, Zambryski *et al.* 1980, Czernilofsky *et al.* 1986a). Once integrated, the DNA seems structurally stable, and is inherited in a normal mendelian fashion (Otten *et al.* 1981, DeBlock *et al.* 1984).

Mapping of transcripts and mutations has defined between 7 and 13 genes on the T-DNA in different Ti plasmid types (see Weising *et al.* 1988). Genes number 1, 2 and 4, the *onc* functions, encode enzymes involved in production of the plant growth regulators auxin and cytokinin in the transformed plant cells.

These compounds are responsible for the sustained tumorous growth of the transformed cells. Another set of genes encode proteins responsible for synthesis of a group of substances called opines. Ti plasmids can be classified according to the type of opines they produce; nopaline and octopine are the best characterised. They are derivatives of arginine which are secreted by the crown gall cells, and support growth specifically of the inciting bacteria.

The first step in development of Ti plasmids as transformation vectors came with the observation that the T-DNA could accommodate additional sequence that was faithfully transferred into the plant genome upon infection (Hernalsteens *et al.* 1980, Holsters *et al.* 1982). Transformed cells were identified by their tumorous phenotype; that is, the ability to grow in culture in the absence of exogenous plant growth regulators. The limitation of this technique was that cells expressing this phenotype clearly could not be regenerated to give mature plants as could normal, untransformed cells. The development of chimaeric genes that conferred transformed plant cells with resistance to normally toxic antibiotics provided an alternative way of selecting transformed cells (Herrera-Estrella *et al.* 1983a,b). Such genes could be inserted into the T-DNA of *A. tumefaciens* and allowed the development of Ti plasmid derivatives that lack the *onc* functions (Zambryski *et al.* 1983b). Tissue explants or even single cells may be transformed with such plasmids, cultured with the required hormones and selected with the relevant antibiotic, eventually to be regenerated into whole, fertile plants. Every cell in the plant is clonally derived from the transformed cell and carries copies of the transferred DNA sequences.

Ti plasmids are too large to manipulate *in vitro*, so sequences of interest were first cloned in small convenient plasmid vectors in *E. coli*, and then mobilised into *A. tumefaciens* where they became inserted into the Ti plasmid by recombination between the 25 bp borders that define the T-DNA (Zambryski *et al.* 1983b, Figure 1.2A). Two observations led to the development of the more convenient binary vector systems; first, none of the sequence normally present between the T-DNA borders is required for T-DNA transfer, and second, T-DNA could be transferred from one plasmid even if the Ti encoded virulence functions were located on a separate plasmid (Hoekema *et al.* 1983). In binary systems the T-DNA is carried by a small plasmid that can replicate in both *E. coli* and *Agrobacterium* (Figure 1.2B). The DNA between the borders is deleted and replaced usually by a selectable chimaeric antibiotic resistance gene and by convenient restriction endonuclease sites adjacent to plant gene regulatory sequences that facilitate insertion and controlled expression of any cloned DNA sequence. The *in vitro* genetic manipulations required to introduce the sequence of interest into the T-DNA are performed with *E. coli* for convenience, then the final plasmid is transferred to *Agrobacterium* by conjugation. The latter carries a modified Ti-plasmid with all the required virulence functions, but no T-DNA.

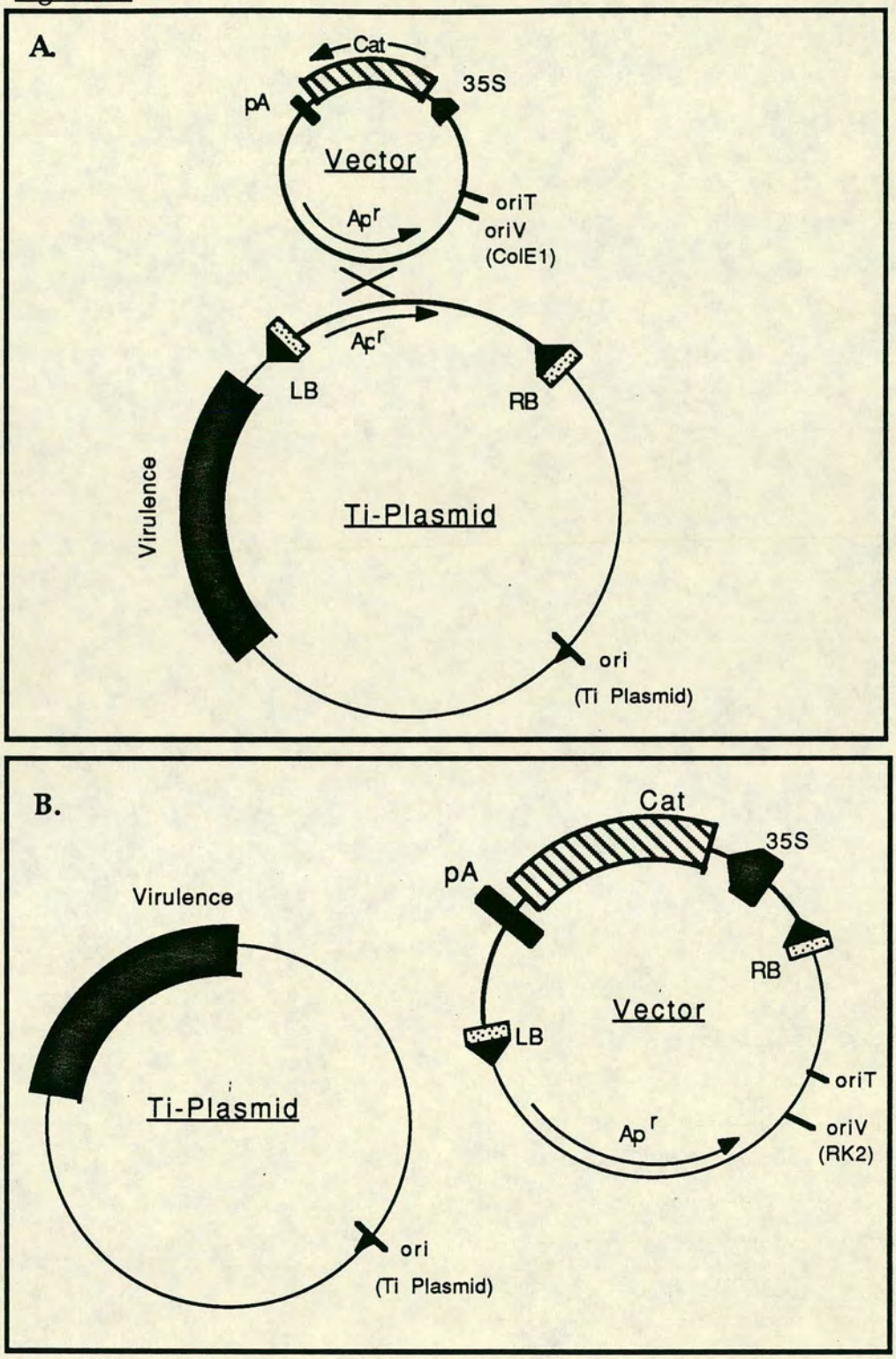
Figure 1.2

Strategies for the Design of Ti-Plasmid Based Transformation Vectors.

A. Integrative transformation vectors. These vectors are small, about 5 kb, and are able to replicate in *E. coli* by virtue of the origin of replication (oriV) usually of the ColE1 type. They contain a marker for selection within bacteria, usually one that encodes β-lactamase (conferring ampicillin or carbenicillin resistance as shown here (Ap^r)) or a neomycin phosphotransferase that confers kanamycin resistance. In addition, they contain a selectable marker for expression in the nuclei of transformed plant cells, such as the one shown here (35 S promoter and polyadenylation signal of the Cauliflower Mosaic Virus directing transcription of a CAT gene). Many vectors also carry a second promoter and polyadenylation signal flanking unique restriction sites to facilitate firstly the insertion of sequences of interest and then their expression after transfer into plant cells. These manipulations can be performed with *E. coli* for convenience, and the final construct can be mobilised into a suitable *A. tumefaciens* recipient by virtue of the origin of transfer (oriT). In *A. tumefaciens* oriV of the vector is inactive and so the vector cannot replicate. However, the Ti-plasmid T-DNA sequence between the 25 bp right and left borders (RB and LB) is replaced by a sequence homologous to the vector. This allows the vector to be integrated between the borders of the Ti-plasmid by homologous recombination, where it is maintained. The Ti-plasmid and chromosomal virulence functions act on the 25 bp borders and the sequence integrated between them, transferring it to plants.

B. Binary Vectors. These vectors generally carry functions similar to those of the vectors described above in all but two respects. The first difference is that the 25 bp T-DNA left and right border repeats are carried on the vector; the nuclear selectable markers and any sequences for insertion and expression of foreign DNA in plant cells are located between them. Secondly, binary vectors carry origins of replication (oriV and oriT) from a wide host range plasmid, usually of the RK2 type as shown here. This allows them to replicate in both *A. tumefaciens* and *E. coli* , and tends to make these plasmids larger (about 10 kb) Thus, following insertion of sequences of interest at the unique cloning sites between the 25 bp borders, the vector can be mobilised into *A. tumefaciens* using *oriT*, where it is maintained by *oriV*. The T-DNA and 25 bp borders of the Ti-plasmid is entirely deleted, thus its virulence functions act only on the vector.

Figure 1.2


A.
Cat
35S
pA
Vector
Ap r
oriT
oriV
(ColE1)
Ap r
LB
RB
Virulence
Ti-Plasmid
ori
(Ti Plasmid)
B.
Virulence
Ti-Plasmid
ori
(Ti Plasmid)
Cat
35S
pA
RB
Vector
LB
oriT
Ap r
oriV
(RK2)

Upon infection of plant cells, the chromosomal and plasmid borne virulence functions act upon the T-DNA region of the cloning vector and mediate transfer of the sequence between the borders into the genome of the recipient (Bevan 1984, Koncz and Schell 1986).

Any cell of a susceptible species that can be induced to divide can potentially be transformed by *Agrobacterium*. In practice, recovery of whole plants is usually desirable, so tissue culture systems that allow plant regeneration from single cells are used; these usually involve regeneration of shoots from the cut edges of leaf pieces, or the development of callus from a single cell protoplast and subsequent regeneration of plants from the callus (Chapter 7). For transformation, sterile leaf pieces or regenerating protolasts are infected with *Agrobacteria* that contain the desired T-DNA sequence. After a few days the bacteria are killed by application of a suitable antibiotic that does not interfere with plant cell growth. Plant tissues are then regenerated in the presence of the antibiotic to which the T-DNA encodes resistance, and those cells that continue to grow are usually transformed. Leaf disc transformation is the most rapid way to generate transformed plants, however protoplast transformation provides the greatest numbers of individual transformants; more than 10^6 protoplasts can be treated at a time, and greater than 1% of these will typically be transformed (usually expressed as a transformation efficiency of greater than 10^{-2}). These systems are discussed more fully in Chapter 7.

Agrobacterium has the disadvantage that its host range is limited, the major crop plants being excluded. If transformed plants are required, a system for regeneration from protoplasts or explants is necessary, but not available for many important species. To extend the range of species that can be transformed, several alternative systems have been developed.

Several viruses that infect plant tissue have received considerable attention as potential vectors. Cauliflower mosaic virus was the first to be used to express a foreign gene in plant cells (Brisson *et al.* 1984). Later, the insertion of cloned viral sequences into T-DNA led to the extension of the *Agrobacterium* host range to maize, in a process called agroinfection (Grimsley *et al.* 1987). Expression of foreign genes cloned in vectors derived from several RNA and DNA viruses has been reported (for example French *et al.* 1986, Takamatsu *et al.* 1987, Töpfer *et al.* 1989). The advantages of viral vectors are their ability to produce systemic infection of mature plants after a localised inoculation, and their independent replication leading to amplification of the cloned DNA (Hayes *et al.* 1988). However, the induction of disease symptoms, their potentially limited capacity to carry foreign DNA sequences, and their failure to transfer the foreign DNA to sexual progeny all limit their routine use as vectors.

More promising alternatives to *Agrobacterium* include various techniques for transferring purified DNA into plant cells. This was first achieved, after many

years of failure, in 1982 by incubating plant cell protoplasts with DNA in the presence of polyethylene glycol and poly-L-ornithine (Draper *et al.* 1982), or after coprecipitation with calcium phosphate (Krens *et al.* 1982). Several modifications to these systems have been made (for example Hein *et al.* 1982 and Pröls *et al.* 1988, Hain *et al.* 1985, Shillito *et al.* 1985), but despite some claims for transformation frequencies of 10^{-2}, usually about one in 10^4 treated protoplasts are transformed; this is up to 1,000 times less efficient than *Agrobacterium*. The advantages are that cloning in specialised Ti plasmid derivatives is not required, there is no host range limitation, RNA can be used instead of DNA, and it is possible to perform transient expression analysis. Transient expression is an alternative to analysis of stable transformants. Following transfer, most cells appear to take up DNA and to express it, though in only few cases does integration of DNA follow (Pröls *et al.* 1988, Chapter 4); transient expression appears to be complete within a few days after transfer, though some of the protein products remain detectable for up to two weeks. This expression can be analysed within a few hours of transformation, usually by assaying the activity of an enzyme encoded by a reporter gene on the transferred DNA, and is able to provide information on the expression and even regulation of genes in plant cells (for example Lipphardt *et al.* 1988). The major limitation of direct DNA transfer in production of stable transformants is that a system for regenerating plants from protoplasts is necessary, currently precluding its application to most cereals. However, reports of plant regeneration from maize and rice protoplasts have been made (Rhodes *et al.* 1988, Abdullah *et al.* 1986 respectively). A second problem is that the DNA is frequently integrated in a severely modified form, perhaps presenting problems for controlled expression of transferred genes (Czernilofsky *et al.* 1986a, Riggs and Bates 1986).

Electroporation is a technique analogous to the chemical treatments described above. Protoplasts are mixed with DNA and subjected to an electric pulse of a few milliseconds or microseconds, depending upon the voltage used, which makes the plasma lemma transiently permeable to macromolecules (Fromm *et al.* 1985, 1986). The discussion above, of the advantages and disadvantages of chemically induced transformation, applies equally well to electroporation.

Microinjection has been successfully used for transformation of tobacco and alfalfa protoplasts (Crossway *et al.* 1986, Reich *et al.* 1986). The technique is most successful when DNA is injected directly into the nucleus, yielding transformation efficiencies of 6 to 20 % of injected protoplasts. Encapsulation of DNA into liposomes has also been used to deliver DNA into protoplasts (Deshayes *et al.* 1985), but transformation frequencies were only 10^{-5}. However, with both of these techniques, the integrated DNA appeared not to have undergone structural modification, perhaps due to its protection from modifying

enzymes in the cytosol (Czernilofsky *et al.* 1986a, Wirtz *et al.* 1987), but they are both dependent on protoplast regeneration systems for recovering transformed plants.

Several techniques for transforming cells other than protoplasts have been developed. De la Peña *et al.* (1987) reported that DNA injected into the juvenile inflorescence of rye can become incorporated into the genomes of some of the progeny. Also, imbibition of wheat, rye and barley embryos with a solution containing DNA led to the transient expression of a bacterial gene in the developing plants (Töpfer *et al.* 1989). Perhaps the most promising technique is the bombardment of tissue under slight vacuum with DNA coated tungsten particles of 1 to 2 μm diameter (Klein *et al.* 1987). The coated particles are propelled by the explosion of a 0.22 rifle charge. Transient expression in maize (Klein *et al.* 1988a), onion and tobacco cells has been achieved, as has stable transformation of soybean root tips and tobacco suspension culture and leaf cells (Klein *et al.* 1988b). This novel technique promises to be of great value, having no obvious host range, and being independent of a protoplast stage, though it does require totipotent cells for recovery of transformed plants.

In all cases above transformation of the nucleus was desired, but the ability to transfer DNA into plant cells potentially provides an opportunity to transform plant mitochondria and chloroplasts. In fact, with the exception of direct microinjection, these transfer techniques are not known to deliver DNA specifically to the nucleus. Probably, they succeed only in transferring DNA across the plasma lemma; little is known of the mechanism by which DNA is transported to the nucleus and integrated into the genome.

1.5 Nucleic Acid Transfer Across Organellar Membranes.

Several forms of nucleic acid transfer across the membranes of mitochondria and chloroplasts have been identified. The first to be considered comprise a series of probably infrequent, isolated events.

1.5.1 Nucleic Acid Transfer During Evolution.

If mitochondria and chloroplasts are indeed derived from endosymbionts that have become degenerate, as discussed above (section 1.1), it is probable that much of the progenitor genome has been transferred to the nucleus. There is good evidence that this type of nucleic acid transfer, from mitochondria and

chloroplasts to the nucleus, is possible; for example all clones from a spinach chloroplast DNA library were found to hybridise with discrete, dispersed nuclear sequences (Timmis and Scott 1983), and maize and yeast nuclear DNA was shown to contain sequences homologous to their respective mitochondrial genomes (Kemble *et al.* 1983, Farrelly and Butow 1983 respectively). Palmer's laboratory has reported that in *Geranium* the *rpoA* gene has been transferred relatively recently during evolution from the chloroplast to the nucleus (Jacobs and Lonsdale 1987). In the latter article the authors argue that the widespread transfer of organellar genes has caused the nucleus to become "progressively subverted to serve the needs of the invading genomes, rather than the intruding organelles having surrendered control of their physiology to the nuclear hegemony".

Whatever the form of the proposed association, if DNA transfer to the nucleus has occurred it seems to have done so on several distinct occasions as mt DNA from different organisms contains slightly different sets of genes; ATPA is apparently encoded on mt DNA of only plants (Hack and Leaver 1983, Isaac *et al.* 1985a, Braun and levings 1985); higher plants and yeast encode ATP9 within mitochondria (Hack and Leaver 1984, Macino and Tzagoloff 1979) whereas in vertebrates, *N. crassa* and *Aspergillus nidulans* it is a nuclear gene product, despite there being a second apparently silent mitochondrial copy of the gene in these fungi (van den Boogaart *et al.* 1982, Brown *et al.* 1984 respectively); the mitochondrial genomes of mammals encode seven subunits of complex I (Chomyn *et al.* 1985), that of *N. crassa* encodes six (Ise *et al.* 1985), and that of yeast, none (Dujon 1983, Grivell 1983). The mitochondria of higher plants differ from each other in the complement of ribosomal protein genes they encode (section 1.3.2).

The proposed transfer of nucleic acid from organelles to the nucleus is in the opposite direction to that required for transformation. Although the hypothesis proposed above is widely accepted, there seems little to directly contradict the alternative interpretation that some mitochondrial genes were simply lost, their roles being assumed either by existing nuclear genes whose products were targeted to mitochondria, or perhaps by transfer of these genes to mitochondria. There is evidence to support several features of this alternative proposal.

Firstly, if mitochondria were once free living organisms, almost certainly many of the functions they required for the free living state, such as those perhaps involved in cell wall or flagellum synthesis, have simply been lost during evolution. Secondly, the results of Marechal-Drouard *et al.* (1988) discussed above (section 1.3.2) imply that the *Phaseolus vulgaris* mitochondria have adopted nuclearly encoded $tRNAs^{leu}$ in place of ancestral mitochondrial ones, though their genes have not been transferred. Thirdly, from the evidence summarised below it seems beyond reasonable doubt that DNA has been transferred to mitochondria during evolution.

Stern and Lonsdale (1982) first reported that maize mitochondrial DNA contains a 12 kb sequence similar to part of the 16 S rRNA gene and a ribosomal protein operon of chloroplasts. A portion of the gene for the large subunit of ribulose bisphosphate carboxylase/oxygenase was also found subsequently (Lonsdale *et al.* 1983). Sequences present in more than one organelle have been termed promiscuous (Ellis 1982), and have been discovered in several species (Schuster and Brennicke 1987b). Schuster and Brennicke (1987a,c) have shown *Oenothera* mitochondria to contain truncated copies of plastid genes for a ribosomal protein and two rRNAs, a complete gene for a plastid $tRNA^{ser}$, and also the first sequence of nuclear origin discovered to have been transferred to a mitochondrial genome: 528 nucleotides homologous to the cytosolic 18 S rRNA. Chloroplast sequences appear to have been transferred into the mitochondrial genomes of six Crucifereae species on three separate occasions (Nugent and Palmer 1988). Once transferred, the sequences appear stable, some having been conserved for over 30 million years despite extensive rearrangements of the genomes in even the most closely related species during the same period. This conservation is remarkable given that in general the transferred sequences appear to have no function in the mitochondria; with the exception of a number of tRNA genes, they have been sufficiently mutated to make retention of their original function doubtful.

Notable exceptions to this generalisation are genes for the maize mitochondrial $tRNA^{trp}$ and $tRNA^{cys}$. As mentioned in section 1.3.1 $tRNA^{trp}$ is located solely on the 2.3 kb linear plasmid. The RNA encoded by this gene and the corresponding gene from wheat are highly homologous to the $tRNA^{trp}$ of bean mitochondria whose sequence was determined by directly sequencing purified RNA (Marechal *et al.* 1987). These share between 96 and 97 % sequence similarity with the corresponding chloroplast tRNAs. Significantly, a sequence with similarity to part of a chloroplast $tRNA^{pro}$ was located about 140 bp upstream of $tRNA^{trp}$ in both wheat and maize mitochondria, almost exactly as they are arranged in wheat chloroplast DNA. The maize $tRNA^{cys}$ is 99% identical to its homologue in wheat chloroplasts, the homology extending into the flanking regions (Wintz *et al.* 1988). These observations strongly suggest that these sequences are of chloroplast origin. Both are transcribed, and the $tRNA^{trp}$ at least appears to be functional in bean mitochondria (Marechal *et al.* 1987). Thus it seems that the intracellular sources of contemporary plant mitochondrial tRNAs and their evolutionary derivation may include all three cellular genomes; some tRNAs appear to be imported from the nucleus, some to be endogenous and the genes for some of these seem to have been derived from chloroplast DNA.

Interestingly, in *Oenothera* mtDNA Schuster and Brennicke (1987a) have discovered a transcribed open reading frame with homology to a reverse transcriptase. They have also observed that all sequences transferred to

mitochondria are transcribed at their proposed site of origin, raising the possibility that promiscuous DNA originates as promiscuous RNA and is integrated into mitochondrial genomes following reverse transcription into complementary DNA. This proposal would be strongly supported by the demonstration that sequences transferred to plant mitochondria include copies of foreign transcripts in processed forms that do not occur in the DNA from which they originate. However sequences homologous to foreign spliced transcripts have not yet been observed in mitochondrial DNA, whereas spacer and intron sequences have been transferred, and genes transcribed from opposite strands of their progenitor DNA appear to have been transferred in a single event (Stern and Lonsdale 1982, Nugent and Palmer 1988).

The existence of promiscuous DNA illustrates a potential for variation in the mitochondrial genome, and suggests that it may tolerate additional sequence acquired after transformation. Interestingly, neither of the highly conserved genomes of chloroplasts and mammalian mitochondria have been shown to contain promiscuous DNA. Sequences derived from chloroplast DNA contribute to, but do not explain, the large variation in plant mitochondrial genome size; in Crucifereae, not more than 6 % of the genome is of plastid origin (Nugent and Palmer 1988), and even if the whole tobacco chloroplast genome (about 150 kb, Shinozaki *et al.* 1986) were transferred to the smallest known plant mitochondrial genome (208 kb, Palmer and Herbon 1987), its size would be only about 60 % of the maize mitochondrial genome (570 kb Lonsdale *et al.* 1984).

1.5.2 Contemporary Nucleic Acid Transfer.

The discovery that nucleic acids, may routinely be transported across mitochondrial membranes is of greater interest. It has already been mentioned that tRNAs are probably transported into the mitochondria of higher plants and of *C. reinhardtii* and *T.thermophila* (section 1.3.2). Three other cases of natural nucleic acid import are described below.

DNA replication in mammalian mitochondria is initiated on an RNA primer synthesised at the replication origin. Near to the point of transition to DNA synthesis the initial transcript is processed by an enzyme called RNase MRP that itself contains an RNA (Chang and Clayton 1987). The sequence of this RNA was determined, and found to be absent from the mitochondrial genome implying that it is imported. Similarly, the primase function that generates the initial RNA primer contains an RNA of either 155 or 160 nucleotides (Wong and Clayton 1986). When the sequence of this was determined, it was found to be identical to the 5.8 S rRNA constituent of 80 S cytosolic ribosomes, a nuclear gene

product, and not encoded on mitochondrial DNA.

Senescence in cultures of *kalilo* strains of *Neurospora intermedia* is associated with the accumulation of defective mitochondrial genomes that contain a 9 kb transposable sequence called kalDNA (Bertrand *et al.* 1985). This sequence is not found in mitochondrial DNA from normal, non-senescent, strains nor in the mt DNA from the pre-senescent cultures of *kalilo* strains. Pre-senescent cultures of the latter strains however do contain a 9 kb linear plasmid homologous to kal DNA existing in free form closely associated with nuclei (Bertrand *et al.* 1986). It is not found in non-senescing strains or, in its free form, in the mitochondria of *kalilo* strains, but in the latter it appears to be transposed into the mitochondria where its insertion into the genome causes senescence.

Though the molecular mechanisms of these postulated transfer events are not understood, they suggest that nucleic acid can traverse the mitochondrial membranes. The specificity of the transfer processes and any macromolecular apparatus that may be involved is not known; however these systems provide potential routes for transformation in their respective species.

1.5.3 Transformation of Organelles.

There have been several reports of experimental organelle transformation in other organisms. The first of these claimed transformation of yeast mitochondria to oligomycin resistance (Atchison *et al.* 1980). A fragment of mitochondrial DNA encoding an oligomycin resistant allele of the ATP6 gene cloned into the yeast 2 μm plasmid was used to transform sensitive yeast spheroplasts. Genetic and statistical evidence for transformation was presented, but no physical evidence has been published.

The second report described transformation of tobacco chloroplasts by *A. tumefaciens* (De Block *et al.* 1985). A bacterial gene encoding chloramphenicol acetyltransferase (CAT, section 3.1.2) expressed from the nopaline synthase promoter of the T-DNA was used. Though this promoter is normally used to achieve expression in nuclei, there was evidence that it would also function in chloroplasts. Maternal inheritance of the resistant phenotype was observed, cell fractionation studies localised transforming DNA and CAT activity to the chloroplasts, and a cotransferred bacterial gene that is not expressed from the nucleus was highly active as expected if it were to be located in the chloroplast (Gruissem and Zurawski 1985). This observation has proven difficult to repeat, suggesting either that sequences transferred to the chloroplast are not stably maintained, or that delivery of DNA into chloroplasts by *Agrobacterium* is not

efficient.

The last four reports all involve use of high velocity tungsten particles to deliver transforming DNA. Boynton *et al.* (1988) reported transformation of *C. reinhardtii* chloroplasts. Cells that were photosynthetically defective due to mutations in one of three chloroplast loci were transformed with cloned wild type alleles. Transformants were recovered by selecting for photosynthetic competence at a frequency of about one in $3x10^6$ treated cells. When analysed by restriction endonuclease digestion and Southern blotting, the chloroplast DNA of the transformants appeared to have been repaired by accurate double recombination or gene conversion events that simply replaced the mutant sequence with that of the wild type allele without introducing additional vector DNA. Photosynthetic competence and the restored form of the allele were inherited in a non-mendelian, usually uniparental fashion. Blowers *et al.* (1989) have repeated these observations and have shown that a chimaeric neomycin phosphotransferase gene can be cotransferred and maintained in the genome even in the absence of positive selection.

In an analogous experiment, Johnston *et al.* (1988) reported restoration of respiratory competence by transforming yeast with cloned sequences that complemented a mutation in the mitochondrial COXI gene. This phenotype was inherited as a mitochondrial gene in sexual crosses. Ethidium bromide stained gels and Southern blots of restriction endonuclease digested mitochondrial DNA revealed the transforming DNA to have replaced mutant sequences by double recombination or gene conversion just as in the case above. One mitochondrial transformant was recovered per 10^7 treated cells, a transformation efficiency 1000 fold lower than that for nuclear transformation by the same technique. Fox *et al.* (1988) were also able to generate mitochondrial transformants by this technique, and showed that in yeast strains lacking mitochondrial DNA the transforming plasmid can become amplified and stably replicated even without direct selection.

Thus it now seems that the use of high velocity microprojectiles coated with DNA may be the best way to transform chloroplasts and mitochondria.

1.6 Conclusion.

A system for genetic transformation of plant mitochondria could lead to a rapid increase in the understanding of mitochondrial biology, while the introduction of CMS into new crops and new varieties is a clear potential application. Several techniques have been developed for introducing DNA into plant cells with the aim of transforming the nuclear genome. However, with the exception of direct microinjection, they are not known to deliver DNA specifically

to the nucleus. Transforming DNA may have several intracellular destinations that perhaps include the chloroplasts and mitochondria. The observations of DeBlock *et al.* (1985) initially suggested that following transformation by *A. tumefaciens* T-DNA could enter the chloroplast, prompting the question addressed in this thesis; does transforming DNA enter the mitochondrion?

If it does, it should be possible to recover a plant with foreign DNA integrated into its mtDNA. There was good reason to believe that the gene constructs used for expression in nuclear transformants would not be effective in plant mitochondria (discussed in section 3.1), thus mitochondrial transformants would previously have been overlooked. The work described in this thesis is directed towards the development of selectable marker genes to allow mitochondrial transformants to be recovered. Chapters 3 to 7 describe the design, assessment and initial deployment of a strategy to recover such a transformant. Chapter 8 evaluates this approach with a discussion of the implications of recent published results and techniques that were unavailable at the outset.

CHAPTER 2.

MATERIALS AND METHODS

2.1. Materials.

2.1.1 Biological Materials.

2.1.1.1 Plants.

Seed from *Nicotiana tabacum* cv. Petit Havana SR1 was a gift from Dr. A.P. Czernilofsky, Max Planck Institut für Züchtungsforchung, D-5000, Köln 30, FRG. A suspension culture of *Nicotiana tabacum* cv. Xanthi was a gift from Dr. M.A. Matzke, Austrian Academy of Sciences, Salzburg, Austria. Tobacco plant JV212 transformed with pCAP212 was a gift from Dr. H.-H. Steinbiß, also of the Max Planck Institut in Cologne.

2.1.1.2 Strains of *Escherichia coli*, and their Genotypes.

JM101	Δ(*lac proAB*) , *thi* ,*Sup E*, [F', *traD* 36, *proAB*, *lacI* q Z ΔM15] (Yanisch-Perron *et al.* 1985).
JM103	Δ(*lac proAB*), *thi* ,*Sup E*, [F', *traD* 36, *proAB*, *lacI* q Z ΔM15], *strA*, *sbcB* 15, *endA*, *hsdR* 4, λ^- (Messing *et al.* 1981).
JM83	Δ(*lac proAB*) , *lacZ* ΔM15, *ara*, *strA*, ϕ80 (Yanisch-Perron *et al.* 1985).
NM522	Δ(*lac pro*), [F'*lacZ* ΔM15], *lacI* q, *hsdR*$^-$*M* (Gough and Murray 1983)
HB101	F$^-$, *recA* 13, *ara*-14, *proA* 2, *lacY* 1, *galK* 2, *rpsL* 2, *xyl*- 5, *mtl*-1, *supE* 44, *hsdS* 20 (r_B^-,m_B^-), λ^- (Manniatis *et al.* 1982).
DH1	F$^-$, *recA* 1, *gyrA* 96, *endA* 1, *hsdR* 17 (r_k^-, m_k^+), *supE* 44, *thi*- 1, λ^- (Hanahan 1983)

2.11.3 Plasmids.

pUC 9	Messing and Vieira (1982)
pUC 18	Yanisch-Perron *et al.* (1985)
pUC 19	Yanisch-Perron *et al.* (1985)
pK 18	Pridmore (1987)
pRT101	Töpfer *et al.* (1987)
pCAP212	Velten and Schell (1985)
pLGV1103	Hererra-Estrella, unpublished, and Czernilofsky *et al.* (1986a)
pDS-5β	Gift from Dr. M. Boutry, University of Louvain, Louvain - la Neuve Belgium
pATP9-1	Gift from Dr. M. Hanson, Cornell University, Ithaca, USA.
pATP S13	Bland *et al.* (1986)
pHSB3	Gift from Dr. P. Isaac, University of Edinburgh.
pIco75	Charles *et al.* (1985b)

2.1.1.4 Bacteriophage M13 Strains

m3a3	Isaac *et al.* (1985b)
mp18	Yanisch-Perron *et al.* (1985)
mp19	Yanisch-Perron *et al.* (1985)

2.1.2 Miscellaneous.

2.1.2.1 Chemicals.

Chemicals were purchased from BDH Chemicals Ltd., Poole Dorset, or from Merck, Darmstadt FRG unless otherwise stated, and were of AnalaR grade. X-gal and IPTG were bought from Northumberland Biologicals Ltd., Cramlington Northumberland. Powdered plant growth media were bought from Imperial Laboratories Ltd. Andover Hants., and plant growth regulators and antibiotics from Sigma Chemical Company Ltd., Poole Dorset.

2.1.2.2 Photography.

Photographs of agarose gels were taken with Ilford HP5 film, developed in Kodak HC110, and fixed with Ilford "Hypam". Plant tissue was photographed with Kodacolour Gold 100ASA. film, which was processed by Colab Ltd., Herald Way, Binley, Coventry.

2.1.2.3 Autoradiography.

Films were either Agfa-Gaevert "Curix", or Kodak XAR-5, and were all developed in an Agfa-Gaevert Gevamatic 60 automatic developer. For autoradiography, films were exposed to Southern blots or chromatograms at -70^{o}C in a Kodak X-Omatic cassette with regular intensifying screens

2.1.2.4 Centrifugation Equipment.

Sorvall GSA, GS3, and SS34 rotors were used in Sorvall RC-5B centrifuges. Bottles of 250 ml and 500 ml were used with the GSA and GS3 rotors respectively. 50 ml polycarbonate tubes, or 15 and 30 ml Corex tubes were used in the SS34 rotor. VTi50, VTi65, and 70Ti rotors were used in Beckmann L8-70 ultracentrifuges. The 70Ti rotor accommodated screw capped polycarbonate tubes of 30 ml, and Beckmann heat sealing tubes were used in the vertical rotors. Occasionally, 14 ml caesium chloride gradients were used for plasmid DNA isolation, in which case a Beckmann 70.1Ti rotor and heat sealing tubes were used with a Sorvall OTD65B ultracentrifuge.

Reactions and manipulations with isolated DNA were performed in Eppendorf or Treff 1.5 ml microcentrifuge tubes which were used in conjunction with Eppendorf 5414, or MSE Micro Centaur centrifuges.

2.2. Construction of Recombinant DNA Molecules *in vitro*, and Cloning in *Escherichia coli*.

2.2.1 Large Scale Preparation of Plasmid and Bacteriophage M13 Replicative Form DNA from *Escherichia coli*.

One millilitre of an overnight culture (section 2.2.9) of a plasmid bearing *E. coli* strain was added to a litre of autoclaved YT (8 g/l DIFCO Bactotryptone, 5 g/l DIFCO Yeast Extract, 5 g/l sodium chloride, pH 7), or LB (10 g/l Bactotryptone, 5 g/l Yeast Extract, 10 g/l sodium chloride, pH 7.2), containing 50 mg ampicillin or kanamycin per litre. Alternatively, 200µl of an M13 infected overnight culture was mixed with 200 µl of a log phase culture of strain JM101, JM103 or NM522 and left at room temperature for 15 minutes. This mixture was used to inoculate 5 ml of YT medium or LB which was shaken for 4 hours at 37°C, and 1 ml then used to infect a litre of the same medium. These cultures were grown shaking at 200 rpm and 37°C for 12 to 16 hours. Cultures were incubated in New Brunswick Shaking incubators.

The bacterial suspension was pelleted by centrifugation (GS3 rotor, 10 minutes at 4°C and 7,500 rpm), and the pellet resuspended in 10 ml of glucose buffer (50 mM glucose, 25 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0). To this suspension were added 1.5 ml of 1.25% w/v lysozyme in glucose buffer, and after 40 minutes on ice, 3 ml of 0.5 M EDTA pH 8.0. Following a further 10 minutes on ice, 15 ml of lysis solution (62.5 mM EDTA, 50 mM Tris HCl pH 7.5, 0.4% SDS) was gently mixed in. This was left for 15 to 30 minutes and then the suspended material was pelleted by centrifugation (70Ti rotor, 90 minutes at 4°C and 45,000 rpm). The supernatant was collected, 0.97 g of caesium chloride per millilitre and 6 ml of 5 mg/ml ethidium bromide were added and the volume made up to 72 ml with CsCl solution (0.97 g of caesium chloride dissolved in 100 ml of water). DNA was banded overnight in two 36 ml gradients formed in a VTi 50 rotor at 45,000 rpm and 20°C. The supercoiled DNA fraction was collected through side puncture and banded again on 5 ml gradients at 60,000 rpm in a VTi 65 rotor at 20°C for four hours.

The DNA was again collected by side puncture, the ethidium bromide removed by repeated extraction with caesium chloride saturated 1-butanol, and the caesium chloride removed by dialysis in LTE buffer (10 mM Tris HCl pH 8.0, 0.1 mM EDTA), according to Manniatis *et al.*. (1982).

DNA was also prepared by a scaled up version of the procedure given in section 2.2.9 in which the cell pellet was resuspended initially in 10 ml, the phenol extraction omitted and, after isopropanol precipitation, the DNA was dissolved in CsCl solution for purification of the supercoiled fraction as above.

The DNA solution was removed from the dialysis tubing and stored frozen

at -20°C, or prior to freezing was concentrated by precipitation in 0.1 M sodium chloride and 70% ethanol (Manniatis *et al.* 1982), and redissolving the pellet in LTE at about 1 µg/µl.

2.2.2 Digestion with Restriction Endonucleases.

Restriction endonucleases were purchased usually from New England Biolabs, Beverly MA. USA., but also from Pharmacia, Bethesda Research Laboratories, Amersham International plc., and Böhringer Mannheim GmbH. Reaction conditions were those recommended by New England Biolabs with the exception that BSA was omitted in reaction volumes below 200 µl. DNA concentration varied between 5 and 100 µg/ml. Reactions conducted above 37°C were under paraffin. When a sample was digested by two enzymes requiring different buffers, low salt or potassium containing reactions were performed first, followed by a four or five fold dilution before the second reaction was initiated.

DNA was either electrophoresed according to section 2.2.3, or if further manipulations were to be performed, it was extracted with phenol and chloroform, and precipitated with ethanol all according to Manniatis *et al.* (1982), and finally was redissolved in LTE after centrifugation.

2.2.3 Agarose Gel Electrophoresis.

Horizontal slab gels of 0.5 to 1 cm depth, and between 25 and 300ml were used. Gels were between 0.8% and 2% (w/v) Seakem Agarose (FMC Corporation, Rockland ME, USA.) in either TAE (40 mM Tris base, 5 mM sodium acetate, 1 mM EDTA Triplex II free acid, and glacial acetic acid to pH 8.0), or TEA (40mM Tris base, 20 mM sodium acetate, 18 mM sodium chloride , 2 mM EDTA Triplex III disodium salt, and glacial acetic acid to pH 8.0), each with 0.1 µg/ml ethidium bromide to stain the nucleic acids.

Gels were immersed in the same buffer and electrophoresed at between 5 and 45 mA until the desired separation was achieved. Nucleic acids were revealed by fluorescence on an ultraviolet TM15 transilluminator (Ultraviolet Products Ltd.) with a peak wavelength of 302 nm at an intensity of 8000 µW/cm^2 at the surface. Photographs were taken using Ilford HP5 film.

The molecular weight of the DNA fragments in each band was estimated using products of *Ava* II and *Hin* dIII digested Lambda DNA, or of *Hae* III digested ϕX174 RF DNA as markers during electrophoresis.

For preparative gels the DNA was extracted with phenol and precipitated

with ethanol (Manniatis *et al.* 1982), before being loaded onto the gel in a ten fold dilution of Loading Dye (50% Glycerol, 0.01%w/v bromophenol blue, 0.01% w/v xylene cyanol, and 50 mM EDTA in TAE). Other samples were loaded directly after adding a one tenth volume of Loading Dye plus 0.1% SDS.

2.2.4 Purification of DNA from Agarose Gels.

Nucleic acid samples were electrophoresed and observed as above (2.2.3). A cut was made in front of the desired band and a piece of NA 45 DEAE membrane (Schleicher and Schuell) was inserted. Electrophoresis was continued until the band was transferred onto the membrane. The membrane was removed, washed with 200 µl of electrophoresis buffer, dried by centrifugation in a microcentrifuge, and incubated for 45 minutes at 65°C in 200 µl of Elution Buffer (10 mM Tris HCl pH 8.0, 1mM EDTA, 1.5 M sodium chloride). The DNA recovered was diluted with 200 µl of water, extracted once with phenol, once with chloroform and then precipitated at -20°C after addition of 1 ml of ethanol. DNA was pelleted in a microcentrifuge, redissolved in 100 µl of 150 mM sodium chloride, and reprecipitated at -20°C with 250 µl of ethanol. The final pellet was dissolved in about 50µl of LTE.

2.2.5 Removal of terminal Phosphates from Linear DNA Molecules.

Böhringer Calf Intestinal Alkaline Phosphatase (24 units/µl) was diluted one hundred fold in 50 mM Tris HCl pH 8.0, 1 mM magnesium chloride, and 1µl was added to about 200 ng of DNA dissolved in the same buffer. The mixture was then incubated for 15 minutes each at 37°C and then at 56°C. A further 1µl of the enzyme was added and the incubations repeated. If the DNA had protruding 5' termini, the incubation was simply at 37°C for 15 minutes. The reaction mixture was extracted twice with phenol, once with chloroform and the DNA was precipitated in ethanol.

2.2.6 Generating Double Stranded Termini on Restriction Endonuclease Digested DNA Molecules that have Single Stranded 5' Termini.

2.2.6.1 Using the Klenow Fragment of DNA Polymerase 1.

About 200 ng of the restriction endonuclease digested DNA was dissolved in 40 µl of Klenow Buffer (10 mM Tris HCl pH 8.0, 10 mM magnesium chloride), with each of the four deoxynucleotide triphosphates at 40 µM, and 6 units of Klenow Fragment (Bethesda Research Laboratories). Incubation was at 37°C for 45 minutes. Between 8 and 10 µl of this reaction mixture were then used directly in ligation as in section 2.2.7, and the rest extracted with phenol, then chloroform and precipitated in ethanol.

2.2.6.2 Using T4 DNA Polymerase.

The polymerisation reaction was performed immediately prior to ligation. A ligation reaction was set up according to section 2.2.7, except that it included the four deoxynucleotide triphosphates at 40 µM, with 3 units of T4 DNA polymerase (New England Biolabs), and the ligase was omitted. This reaction was incubated at 37°C for 10 minutes, then the ligase was added and ligation continued as in section 2.2.7.

2.2.7 Ligation of DNA Molecules.

DNA prepared according to sections 2.2.2, 2.2.4 or 2.25 was resuspended in 20 to 40 µl of ligation buffer (50 mM Tris HCl pH 7.4, 10 mM magnesium chloride, 10 mM DTT, 1 mM ATP) with 1 unit of DNA ligase (New England Biolabs or Bethesda Research Laboratories). Vector and insert were included at an estimated molar ratio of between 1 : 2 and 1 : 4. The reaction was performed usually from 15°C to room temperature, or for blunt end ligations, for 4 hours at room temperature. Ligation products were used to transform *E. coli* as described below.

2.2.8 Preparation and Transformation of Competent Bacterial Cells.

Overnight cultures of *E. coli* strains NM522, HB101, and JM101 were diluted 100 fold in 100 ml of LB in a 250 ml conical flask, and grown for 90 minutes shaking at 300 rpm and 37°C. The cells were pelleted in sterile tubes at 0°C and 2000 rpm in a MSE LR4 centrifuge for 10 minutes. The pellet was resuspended in 50 ml of ice cold 0.1 M magnesium chloride, pelleted again, and gently resuspended in up to 2 ml of ice cold 0.1 M calcium chloride, then left on ice for between 2 and 24 hours.

Transformations were performed for 40 minutes on ice with about 25 ng of DNA, diluted at least five fold in 75 mM calcium chloride, added to 200 µl of competent cells. The cells were heat shocked at 42°C for 2 minutes, and returned to ice.

For recovery of plasmid bearing transformants, the cells were incubated with 1 ml of LB for 1 hour at 37°C. They were concentrated by centrifugation, and spread on LB which had been solidified with 1.2% (w/v) DIFCO Bacto Agar, and which contained 50 µg ampicillin per ml or kanamycin. β-galactosidase activity was assayed by including X-gal at 40 µg/ml. Potential recombinants were identified as white colonies, owing to their inability to breakdown X-gal to a blue product.

For recovery of recombinant M13 phage, the transformed cells were mixed with 200 µl of an early log phase culture of the same strain,10 µl 0.1 M IPTG, 50 µl of 2% (w/v) X-gal dissolved in dimethyl formamide, and 3 ml of YT or LB with 0.8% (w/v) agar at 42°C. This mixture was spread over YT or LB solidified with 1.2% (w/v) agar. All plates were incubated at 37°C for 12 to 16 hours.

Competent cells of *E. coli* strains JM83, DH1, and JM103 were prepared as follows (modified from Hanahan 1983). An overnight culture was diluted 100 fold in K Broth (0.5% (w/v) yeast extract, 2% (w/v) tryptone, 0.4% (w/v) magnesium sulphate, 10 mM potassium chloride, pH 7.6) and grown by shaking at 37°C and 300 rpm to an optical density at 550 nm of about 0.3. The optical density was determined, multiplied by 5/0.3 ml to give the amount of this culture to add to 100ml of warmed K Broth. This culture was grown as above to an optical density of 0.5 ± 0.02. The cells were pelleted at 4°C and 4000 rpm for 5 minutes in a Heraeus Christ GL minicentrifuge, resuspended in 7.5 ml of TFB1 (30 mM potassium acetate, 50 mM manganese chloride, 100mM rubidium chloride, 10 mM calcium chloride, 15% glycerol and pH 5.8), pelleted again, and resuspended finally in 1 ml TFB2 (10mM sodium MOPS pH 7.0, 15 mM calcium chloride, 10 mM rubidium chloride, and 15% glycerol). Cells were stored frozen at -70°C before use. Transformations were as above but with 50 µl of cells in a final volume of 70 µl and for 30 minutes.

2.2.9 Analysis of Recombinant Plasmids and Phage

A sterile toothpick was inserted into the centre of a bacterial colony or phage plaque, and used to inoculate 5 ml of YT or LB containing antibiotic where appropriate. These were grown as in section 2.2.1.

Supercoiled DNA was extracted by a modification of the method of Birnboim and Doly (1979). Cells from 1.5 ml of culture were pelleted in a microcentrifuge tube, resuspended in 100 µl of Glucose Buffer (25 mM Tris HCl pH 8.0, 10 mM EDTA, 50 mM glucose and 1 mg/ml lysozyme) and incubated on ice for 10 minutes. 200 µl of 0.2 M sodium hydroxide with 0.2% (w/v) SDS was gently mixed in, and left on ice for 5 minutes, after which 150 µl of an acidic potassium acetate solution (60 ml of 5 M potassium acetate plus 11.5 ml of glacial acetic acid and 28.5 ml of water) were added, and the insoluble material pelleted in a microcentrifuge. The supernatant was extracted with a 1 : 1 mixture of phenol and chloroform, and the nucleic acid precipitated at -20°C with 280 µl of isopropanol. After centrifugation the pellet was redissolved in 200 µl of 0.1 M sodium acetate in 50 mM Tris HCl pH 8.0, and precipitated at -20°C with 1 ml of ethanol. The precipitate was again pelleted washed with 70% v/v ethanol, and redissolved finally in 50µl LTE buffer. Between 5 and 10µl of this was used for digestion with restriction endonucleases, as in section 2.2.2, but with 5 to 10 µg of ribonuclease A includeds during the last 10 minutes of digestion.

2.2.10 Determination of the Nucleotide Sequence of DNA.

2.2.10.1 Preparation of Single Stranded M13 DNA.

A 5 ml overnight culture was grown and cells pelleted as described in section 2.2.9. From the supernatant 1.2 ml was removed and centrifuged again, then 1 ml of this supernatant was mixed with 250µl 20% (w/v) PEG in 2.5 M sodium chloride and left at room temperature for 30 minutes. Phage were pelleted in a microcentrifuge, and all the supernatant removed. The phage were resuspended in 100 µl of LTE, mixed with 50 µl of phenol, and 5 minutes later with 50 µl of chloroform. After centrifugation, 80 µl of the upper aqueous phase was removed, mixed with 120 µl of water and precipitated as usual with ethanol (Manniatis *et al.* 1982). The DNA was dissolved finally in 50 µl of water.

2.2.10.2 DNA Nucleotide Sequencing Reactions.

Sequencing reactions followed the Sanger dideoxynucleotide chain termination method (Sanger*et al.* 1977, 1980). The polymerised DNA was labelled using [α^{35}S]-dATP analogue (specific activity 1000 Ci per mMol.) from Amersham International plc. The sequencing primer was 5'TCCCAGTCACGACGT 3'. Gels were 8% polyacrylamide (8% w/v acrylamide, 0.25% w/v bisacrylamide, 8 M urea 0.1% w/v ammonium persulphate, 0.005% v/v TEMED in TBE [Manniatis *et al.* 1982]) and were 300 x 400 x 0.4 mm in size. They were run at 65 W constant power for between 2 and 6 hours, fixed for 10 minutes in 10% glacial acetic acid, transferred onto Whatman 3MM paper and dried for 2 hours using a Zabona AG (Basel) gel drier. The gel was exposed to Kodak X-Ray film between lead plates at room temperature for up to 7 days.

2.3. Transfer of DNA into Plant Cells.

Several tissues and protocols have been used for stable or transient transfer of DNA to plant cells. Only the protocols which were finally adopted and then routinely used are presented below. Any relevant variations employed in their development, and any alternative protocols that were tried are described in the Results section.

2.3.1. Plant Tissue for Transformation

2.3.1.1 Growth of Aseptic Tobacco Plants

Seeds from self pollinated *Nicotiana tabacum* cv. Petit Havana SR1 (Maliga *et al.* 1975) were surface sterilised by immersion for 2 minutes in 70% ethanol, then for 10 minutes in 14% (w/v) hypochlorite. 100% hypochlorite has 10 to 14% available chlorine. The seeds were then washed in three changes of sterile distilled water, and left for 10 minutes to dry. Three seeds were placed in a 1L glass preserve jar containing 150 to 200 ml MS medium (Murashige and Skoog 1962, and supplied by Imperial Laboratories) made up to half the recommended concentration and without hormones, solidified with 0.8% agar (DIFCO Bacto-Agar). The jars were incubated at 24 to 26^{o}C with 16 hours of light at 1000 to 3000 Lux per day. Plants were grown until they almost reached the top of the jar, which took about 10 weeks.

If the plants had not been used up to this point, the top shoots were removed

from below the second leaf, transferred to a fresh jar containing the same medium, and grown as above. After three or four subcultures, the plants were thrown away and new material grown from seed.

For transformation the apical shoot and all but the lower of the leaves were used. After 1 to 2 weeks, new shoots had grown in the leaf axils, and were large enough to be subcultured as above.

2.3.1.2. Growth of Suspension Cultures of *Nicotiana tabacum* cv. Xanthi

A suspension culture was maintained by shaking at 100 rpm and 25°C in Murashige Minimum Organic Medium (Imperial Laboratories) supplemented with 0.5 mg/L 2,4-D and adjusted to pH 5.8 with 0.5 M potassium hydroxide.

At four or five day intervals, 10 ml of cells and medium were pipetted into 30 ml of fresh medium. Each month the suspension was filtered, and all cells passing through a 0.3 mm sieve but retained on a 0.1 mm sieve were added to 30 mls of fresh medium. This prevented the culture from developing large clumps of cells which were difficult to digest during protoplast isolation.

2.3.2 Isolation of Protoplasts.

2.3.2.1 Protoplasts from Leaf Tissue.

This was performed by a modification of the procedure described by Nagy and Maliga 1976. To prepare between 5 $x10^6$ and 1.5 $x10^7$ mesophyll protoplasts, three tobacco plants grown for 6 to 8 weeks as described in section 2.3.1.1 were used. The shoots and all but the lower of the leaves were cut into pieces of about 0.5 cm square and placed into 30 to 40 ml of enzyme solution (1% (w/v) cellulase "Onozuka R10", 0.5% (w/v) Mascerozyme R10 [both Serva, Heidelberg FRG.], dissolved in K3 0.4 medium [K3 medium supplemented with 0.4 M sucrose; Nagy and Maliga 1976, Wullems *et al.* 1981], adjusted to pH 5.6 with 0.5 M potassium hydroxide, and sterilised by filtration through a 0.22 µm membrane). The leaf pieces were left for 12 to 16 hours in this solution at room temperature.

The contents were gently shaken for 5 to 10 minutes to release the protoplasts, which were pipetted off in the enzyme solution and sieved through meshes of 0.3 mm and 0.1 mm. Intact protoplasts were further purified by centrifugation for 10 minutes at 100 X g (Hettich-Universal 2S centrifuge), causing them to float and separate from the cell debris which pelleted. This was sucked off

with a glass capillary, and the protoplasts resuspended in 20 ml K3 0.4, 12 µl were removed for counting in a Neubauer haemocytometer, and the rest centrifuged again to remove the final contaminants. It was found that repeating this washing step often led to improvement in regeneration of the protoplasts and was included if the protoplasts had a tendency to form buds or were slow to initiate division.

After the final wash the protoplasts were resuspended in one of several different media, depending upon the method of transformation to be used. These are described in sections 2.3 and 2.4.

2.3.2.2 Protoplasts from suspension cells.

The protocol described below for digestion of cell walls is derived from that of Uchimaya and Murashige 1974. *Nicotiana tabacum* cv. Xanthi cell suspensions grown as described in section 2.3.1.2 were used 3 or 4 days after subculture. 30 ml of culture was centrifuged at 100 X g, and the supernatant pipetted off leaving a cell pellet which was resuspended in 10 times its weight of enzyme solution (1% (w/v) Cellulase "Onozuka R10", 0.2% (w/v) Mascerozyme R10 in 0.7 M mannitol, pH 5.7) and agitated on a gyrotory shaker at 50 rpm and 25°C.

Digestion was continued for 4 to 5 hours or until individual spherical protoplasts could be seen by using a microscope. The digestion products were sieved through a 0.3 mm and a 0.1 mm mesh, then pelleted by centrifugation at 100 X g. Protoplasts for transformation were further purified by flotation on 0.6 M sucrose, 3 mM MES pH 5.7 and then on K3 0.4, both at 100 X g.

This procedure yields over $1x10^7$ protoplasts which are viable for transient expression but which have never been observed to divide, perhaps owing to the age of the culture.

2.3.3 Transfer of DNA into Protoplasts for Transient Gene Expression.

Using chemical and electrical means, protoplasts can be induced to take up DNA from solution and to express it for a number of days at a level that often can be easily measured (e.g. Fromm *et al.* 1985, Pröls *et al.* 1988). Two techniques have been successfully used in this work.

2.3.3.1 DNA Uptake Induced By PEG and Calcium Nitrate.

This technique is a modification of a procedure developed by Hein *et al.* (1982) for protoplast fusion in which DNA is included in a fusion reaction induced by PEG, a high calcium ion concentration and a high pH. Both Wirtz *et al.* (1987) and Pröls *et al.* (1988) have used the procedure successfully.

After isolation according to the protocols in section 2.3.2, the protoplasts were resuspended in K3 0.4 to a concentration of 1 $x10^6$ per 240 µl. For each sample to be transformed, 240 µl of this suspension were carefully pipetted into the centre of a 9 cm petri dish. Between 20 and 40 µg of plasmid DNA dissolved in 45 µl of LTE (10 mM Tris HCl pH 8.0, 0.1 mM EDTA) was sterilised by being vigorously shaken in a sealed tube with 40 µl of chloroform and then spun for 15 minutes in a microcentrifuge. The protoplasts were mixed very slowly with 40 µl of this solution, and then with 200 µl of PEG solution (0.1 M calcium nitrate, 0.45 M mannitol, 25% (w/v) polyethylene glycol 6000 [BDH Ltd.], adjusted to pH 9 and filter sterilised), and left for 20 minutes.

Taking care to disturb the protoplasts as little as possible, 5 ml of wash solution (0.275 M calcium nitrate, adjusted to pH 6 with potassium hydroxide and autoclaved) was added dropwise to the mixture. The protoplasts appeared particularly sensitive to even slight disturbance when the wash solution was first added.

The washed protoplasts were allowed to stand for 10 minutes before being pelleted at 50 to 100 X g, and were then resuspended in 2.5 ml of K3 0.4. The number of spherical protoplasts remaining was estimated with a haemocytometer, and the suspension then diluted to about 1 $x10^5$ per ml with K3 0.4 if necessary. Survival rates were generally between 30 and 50% of the initial number. Transformed protoplasts were incubated in the dark for 24 to 72 hours at 26°C before being assayed for expression of the transferred DNA (section 2.4.1).

2.3.3.2 DNA Uptake Induced By An Electric Field

This technique is generally called electroporation and the protocol described below is taken from Caplan and Dekeyser (1987), and Fromm *et al.* (1985). The electric pulse was generated by a Bio Rad GenePulser.

Protoplasts prepared according to section 2.3.2 were resuspended to a known density of between 5 $x10^5$ and 1 $x10^6$ per 800 µl in filter sterilised electroporation buffer (10 mM HEPES pH 7.2, 0.4 M sucrose, 4 mM calcium chloride). Plasmid DNA was sterilised as in section 2.3.3.1 and pipetted with 800 µl of protoplast suspension into the electroporation cuvette supplied with the pulse generator. The electrodes consisted of 1 cm wide aluminium strips set 0.4 cm apart against

opposite sides of the cuvette. The cuvette was sealed and allowed to stand on ice for 10 minutes. A relatively long, low voltage pulse with exponential decay (Fromm *et al.* 1985) was generated by setting the capacitance of the machine to 25 μF, and its output voltage to 200 V. Under these conditions, a peak field strength of 500 V/cm with a half life of decay of about 10 ms was delivered to the sample. The cuvette was returned to ice for 10 minutes, then the sample was transferred to a petri dish, diluted to 5 ml with K3 0.4 and incubated for between 24 and 48 hours in the dark before being assayed for expression of the transferred DNA (section 2.4.1)

2.3.4 Stable transformation of Plant Cells.

Following transfer to plant cells, some fraction of the DNA becomes stably integrated into the genome of the cell where it can confer a selectable phenotype (for example Hain *et al.* 1985, Shillito *et al.* 1985, Czernilofsky *et al.* 1986a).

2.3.4.1 Transformation of Tobacco Protoplasts.

This was performed exactly as described in section 2.3.3.1 for transient expression using the PEG-calcium nitrate procedure with tobacco mesophyll protoplasts.

2.3.4.2 Cultivation of Transformed Calli.

After three days in the dark, the protoplasts had begun to generate cell walls and to elongate, at which point they were transferred to low light (less than 1000 Lux, either continuous or 18 hour day) at 26°C and left for a further 4 days. The protoplast suspension was divided into aliquots of 2.5 ml in 5 cm petri dishes and mixed with an equal volume of 1.2% agarose solution. To make this solution, 0.3 g of Seaplaque low gelling temperature agarose (FMC BioProducts, Rockland ME USA) was firstly autoclaved in a small bottle. Then 17 ml of K3 0.4 (2.2.1) was mixed with 8 ml of K3 0.1, which is identical to K3 0.4 except that it has a sucrose concentration of 0.1 M, and this solution is filtered through a 0.22 μm membrane into the autoclaved agarose. This was dissolved by placing the bottle in a boiling water bath, resulting in a solution of 1.2% (w/v) agarose in K3 medium with 0.3 M sucrose. This solution was cooled to below 37°C in a water bath. After mixing

the protoplasts with the agarose the dishes were sealed and left overnight.

The solidified agarose "bead" was divided into 4 or 6 segments which were transferred to a 9 cm petridish, and 10 ml of K3 0.3M sucrose were added. Each week the medium was replaced and the sucrose concentration reduced by 0.05 M with each change, until after 5 weeks a concentration of 0.1 M was reached, at which the culture was maintained. During the first four weeks the calli grew to become small but visible calli in the bead.

2.3.4.3 Selection of Transformed Cells

In the presence of several antibiotics including kanamycin and chloramphenicol, growth of the protoplasts into callus is inhibited unless the cells express a suitable resistance marker. Cells expressing such a marker can therefore be selected during the culture phase.

For selection of kanamycin resistant calli, kanamycin acid sulphate (Sigma) was dissolved in water at 50 mg/ml and stored at -20°C. It was added to the protoplast culture media at a final concentration of 50 µg/ml. Selection was applied directly after the protoplasts were embedded in agarose, and was maintained throughout the growth of the culture. Transformants were first seen 3 or 4 weeks after selection was initiated, and continued to appear for up to 8 weeks.

For selection of chloramphenicol resistant calli, chloramphenicol (Sigma) was dissolved in water at 2 mg/ml and stored at -20°C. The solution was shaken and warmed after thawing to ensure that the antibiotic was fully dissolved.

After embedding in agarose, the protoplasts were grown for 1 week in the absence of selection. Chloramphenicol was added for 2 weeks at 40 µg/ml, then at 10 µg/ml, and then the cells were again left without selection. During this period the first transformants appeared as large bright green calli amongst the small, paler sensitive calli. Chloramphenicol was periodically replaced for 2 weeks at a time at 10 µg/ml to prevent the background calli from overgrowing the agarose beads. New transformants continue to appear up to three months after protoplast isolation.

2.3.4.4 Maintenance of Transformed Calli.

When the calli were about 4 mm in diameter they were transferred to petri dishes containing MS medium (Murashige and Skoog 1962) solidified with 0.8% (w/v) agar and supplemented with 1 µg/ml NAA, 0.2 µg/ml Kinetin, and either

50 µg/ml kanamycin or 10 µg/ml chloramphenicol. Powdered medium was bought from Imperial Laboratories, dissolved as instructed and autoclaved with the agar (DIFCO Bacto-Agar). Hormones and antibiotics were filtered in through a 0.22 µm membrane when the medium had cooled to below 50°C.

Shoots were induced on the calli by using 0.5 µg/ml BAP and 0.1µg/ml NAA in place of the hormones used above. After about four weeks shoots could be seen and were removed to half strength MS medium without hormones but with either 10 µg/ml chloramphenicol or 50 µg/ml kanamycin. In this medium they developed roots and were grown as described in section 2.3.1.1.

2.4. Analysis of Transformed Plant Tissue.

2.4.1 Detection of Chloramphenicol Acetyl Transferase Activity in Plant Tissue.

After transformation according to section 2.3.3, the protoplasts were mixed with an equal volume of North Sea water diluted to 600mOsm, transferred to centrifuge tubes and pelleted at 100 X g. All but 1 ml of the supernatant was pipetted off, and the remainder transferred to a microcentrifuge tube along with the protoplasts which were pelleted. The cell pellet was frozen in liquid nitrogen, and either analysed immediately or stored at -20°C. If callus or leaf tissue was to be assayed, about 50 mg was weighed transferred to a microcentrifuge tube, and frozen as above.

The following assay procedure is adapted from Gorman *et al.* (1982). The frozen tissue was resuspended in 50 µl of 250 mM Tris HCl pH 7.5, was broken by grinding with a glass rod for 30 seconds, and then placed at 65°C for 10 minutes. After cooling on ice, 1 µl of 50mM acetyl coenzyme A, and 0.5 µCi of ^{14}C-chloramphenicol (Amersham International plc., CFA-515) were added. This mixture was incubated at 37°C for 1 hour, and then extracted three times with ethyl acetate. The organic phases were pooled and the solvent evaporated off at 95°C for 5 minutes. The sample was redissolved in 20 µl of ethyl acetate, and applied to a 0.2 mm silica gel thin layer chromatography plate (Merck). The sample was fractionated by chromatography in a 19 : 1 mixture of chloroform and methanol respectively. The chromatogram was air dried and exposed to Kodak XAR film for 12 to 36 hours.

2.4.2 Detection of Transforming DNA in the Plant Genome.

2.4.2.1 Isolation of Total Genomic DNA.

Plant DNA was isolated according to Czernilofsky *et al.* (1986a). Between 10 and 15 g of leaf or callus material was ground in liquid nitrogen with a pestle and mortar. The resulting powder was kept frozen, and transferred to a 30 ml centrifuge tube where an equal volume (w/v) of boiling Extraction Buffer (2% (w/v) CTAB, 0.1 M Tris HCl pH 8.0, 10 mM EDTA, 1.4 M sodium chloride) and 1 ml of β-mercaptoethanol were added. The sample was heated to 50°C and mixed thoroughly with an equal volume of a 1 : 24 mixture of isoamyl alcohol and chloroform. The phases were separated by centrifugation in an SS34 rotor for 20 minutes at 7,500 rpm and 20°C. The upper phase was recovered, a one tenth volume of 10% (w/v) CTAB in 0.7 M sodium chloride was added, and the chloroform extraction repeated as above except that the rotor was spun at 12,000 rpm. The upper phase was removed, and the nucleic acids precipitated out by adding a one tenth volume of precipitation buffer (1% [w/v] CTAB, 50 mM Tris HCl pH8.0, 10 mM EDTA). After 30 minutes at room temperature, the precipitate was pelleted by centrifugation at 12,000rpm for 20 minutes and redissolved for 1 hour in CsCl Solution (97 g of caesium chloride added to 100 ml of 50 mM Tris HCl pH 8.0, 5 mM EDTA, 10 mM sodium chloride). The DNA was banded and recovered as described for supercoiled DNA in section 2.2.1.

2.4.2.2 Southern Blot Analysis.

Southern blots (Southern 1975) were made according to Hain *et al.* (1985), and Hughes *et al.* (1978). Ten micrograms of genomic DNA was cleaved for 4 hours in 300 µl reactions with 200 units of restriction endonuclease, as described in section 2.2.3. Digested DNA was electrophoresed through 1% (w/v) agarose gels in TEA as described in section 2.2.3. Gels were immersed for 1.5 hours, first in Denaturation Buffer (1.5 M sodium chloride, 0.5 M sodium hydroxide) and then, after two washes in water, in Neutralisation buffer (0.5 M Tris, 1.5 M sodium chloride, pH 7.5). DNA was transferred to nitrocellulose membranes (Schleicher and Schuell) in 6 x SSC (20 x SSC is 3 M sodium chloride, 0.3 M sodium citrate) by capillary blotting for 16 hours. Filters were baked for 2 hours at 80°C under vacuum, and prehybridised by shaking at 42°C for at least 16 hours in Annealing Mix (50% (v/v) deionised formamide, 25 mM phosphate buffer pH 7.4, 0.2% (w/v) each of Ficoll 400, polyvinyl pyrrolidone and BSA (10x Denhardts solution), 3 x

SSC, and 500µg/ml salmon sperm DNA). The annealing mix was removed and the filter probed as described in section 2.4.2.3. After hybridisation, the filter was washed three times in 0.1% (w/v) SDS, 0.1 x SSC for up to 1 hour at 50°C. Washed filters were dried and exposed to Kodak X-Ray film at -70°C with intensifying screens (Bonner and Laskey 1974).

2.4.2.3 Generating Radioactive Hybridisation Probes.

Plasmid DNA or an isolated fragment of cloned DNA was labelled either by nick translation (Rigby *et al.* 1977), or by oligonucleotide random priming (Feinberg and Vogelstein 1984).

Nick translations were performed using the Amersham International Nick Translation Kit with 40 ng of DNA, and 50 µCi of α-^{32}P-dCTP (specific activity 15.17 TBq/mmol, Amersham International plc.) for 90 minutes. Random priming was performed as described by Feinberg and Vogelstein but with purified DNA fragments as well as fragments still in agarose.

Unincorporated nucleotides were removed following nick translation by running the reaction products through a Sephadex G50 column in a pasteur pipette. The Sephadex was equilibrated in G50 Buffer (150 mM sodium chloride, 10 mM EDTA, 0.1% (w/v) SDS, 50 mM Tris HCl, pH 7.5). The sample was loaded in 100 µl and after it had run into the column, was washed through with about 1 ml of G50 Buffer. Fractions of about 10 drops were collected in microcentrifuge tubes, monitored for radioactivity, and the initial fractions from the first peak were pooled.

Unincorporated nucleotides were removed after random priming by filling a 1 ml plastic syringe to the 0.8 ml mark with Sephadex G50 (equilibrated with LTE) over glass wool. Buffer was repeatedly added to the top of the column which was then centrifuged at 2,500 rpm for 90 seconds in a BTL bench centrifuge until all the added volume was recovered. The probe mixture was loaded onto the column in 200 µl and the DNA recovered by centrifugation for 150 seconds at 3000 rpm.

The probes were denatured for 20 minutes in a boiling water bath, and were then pipetted directly into 10 to 15 ml of Annealing Mix. Hybridisation was for 48 hours under the same conditions as the prehybridisation.

CHAPTER 3

VECTORS FOR MITOCHONDRIAL TRANSFORMATION

3.1 Introduction.

Transformation in most systems is a rare event. Bacterial transformation typically yields one transformant per thousand treated cells per microgram of DNA when highly efficient procedures are used (for example, see section 2.2.8). About 10^{-3} treated mammalian cells and between 10^{-7} and 10^{-4} treated yeast cells (Shapira *et al.* 1983, Hinnen *et al.* 1978, Beggs 1978 respectively) are transformed per microgram of DNA. When plasmid DNA alone is used, plant transformants are typically recovered at a frequency of 10^{-4} and 10^{-5} treated cells per microgram (Paszkowski *et al.* 1984, Hain *et al.* 1985) though *A. tumefaciens* generally transforms between 1 and 25% of available cells. In only the latter case, with such high transformation frequencies, is it feasible to detect transformants by simply screening clones for the foreign DNA, and even this task is easier if the transferred DNA carries a 'screenable' gene whose product is easily assayed. In the other cases transformation must be performed with a selectable marker.

A selectable marker is a gene whose expression will allow a transformant to survive conditions that a non-transformant cannot. Most frequently these genes complement a mutation to auxotrophy, for example in thymidine or leucine metabolism (Bandyopadhyay and Temin 1984, Hinnen *et al.* 1978 respectively), or encode resistance to an antibiotic to which the cells are normally sensitive (Bolivar 1978, Herrera-Estrella *et al.* 1983a and b). Usually, following transformation, the treated cells must be cultured to allow multiplication before individual clones can be analysed, and selection can conveniently be applied at this stage; the screen is thus for survival. In this way the number of cells that can be analysed depends only upon the number that can be transformed and cultured, which for the cases described above is frequently greater than 10^6. Those that survive the selection procedure are likely to be transformants, and this can be confirmed by more direct genetic or biochemical analysis.

As transformation of yeast mitochondria and, potentially, plant mitochondria is even less efficient than nuclear transformation, it was decided

that a selectable marker for this event would probably be necessary. Although knowledge of plant mitochondrial gene expression and inheritance mechanisms was limited, enough was known to make a worthwhile attempt at constructing such a selectable marker. The initial part of the work described in this thesis was therefore aimed at the design of a gene whose expression would confer a selectable phenotype to plant cells, but which would be expressed only if situated in their mitochondria.

3.1.1 The Choice of Plant Species for Transformation.

Several monocot and dicot species have been transformed. The mitochondrial molecular genetics of *Zea mays* had been described in most detail when this work was initiated, however this species had not been transformed, and even now its transformation is not routine. As mitochondrial transformation may be a rare event, and as several transformation techniques may be used in the course of the work a species for which high transformation rates can be achieved, and one that is amenable to the full range of techniques was desired. Tobacco has both these attributes, *Nicotiana tabacum* being most frequently used to develop new transformation techniques. This owes largely to its ease of manipulation in the various tissue culture systems that are involved in most of the transformation procedures used to date. *N. tabacum* cv. Petit Havana SR1 (Maliga *et al.* 1975) was thus chosen for mitochondrial transformation, despite there being only limited knowledge of the molecular genetics of its mitochondria.

3.1.2 The Choice of Selectable Marker.

When this work was initiated, four systems had been used in nuclear transformation experiments for selection of transformed plant cells. These were resistance to either methotrexate, the aminoglycoside antibiotics kanamycin and hygromycin, or chloramphenicol.

Methotrexate inhibits dihydrofolate reductase, and is used widely to select mammalian cells transformed with a resistant form of the enzyme. However selection of transformed plant cells using this system was difficult and was not recommended (Herrera-Estrella *et al.* 1983a). Kanamycin is a potent inhibitor of plant cell growth. Effective resistance to kanamycin is conferred to transformed plant cells by the neomycin phosphotransferase enzymes (NPT) which are

encoded by the NPT genes (*nptI* and *nptII*) of the bacterial transposable elements Tn*903* and Tn*5* respectively (Pietrzak *et al* 1986, Herrera-Estrella *et al.* 1983a). These genes thus provide a convenient selection system, with *nptII* of Tn*5* being most frequently used. The hygromycin selection system appeared similarly effective, but having been only recently developed, it had not been widely evaluated (van den Elzen *et al.* 1985). Selection with chloramphenicol is more problematical. Resistance is conferred by the enzyme chloramphenicol acetyltransferase (CAT). The CAT gene (*cat*) from Tn*9* of *E. coli* has been used to select nuclear transformants (DeBlock *et al.* 1984), though only partial resistance was conferred. At the low chloramphenicol concentrations that had to be used for selection, the growth of untransformed cells was not completely inhibited, making it more difficult to distinguish the transformants amongst them.

Despite the advantages of the neomycin and hygromycin transferase genes for selection of nuclear transformants, they may not be well suited for selection within mitochondria. Kanamycin binds to the small ribosomal subunit and can either induce misreading of the mRNA or inhibit translation, depending upon the system and the conditions used. It is often assumed that because kanamycin interferes with bacterial translation, it acts in plant cells on organellar ribosomes as these have some prokaryotic features. However, this assertion has not been rigorously tested, and the literature reveals that kanamycin is active upon the 80S cytoplasmic ribosomes of eukaryotes from several disperse phyla (Table 3.1). Significantly, this includes wheat ribosomes on which kanamycin potently induces misreading of the mRNA but does not appreciably inhibit translation (Wilhelm *et al.* 1978b). Similar studies have not been undertaken with tobacco, but the widespread activity of kanamycin on the 80S ribosomes of other species suggest that it is likely to have a similar effect in tobacco. If NPTII activity were to be located in the mitochondria, the cytosolic 80S ribosomes may not be efficiently protected. It is arguable that in such a situation, given adequate intracellular diffusion of kanamycin, sufficient inactivation may occur to allow discrimination of transformants. However, investigation of a claim by Davey *et al.* (1970) that mitochondrial ribosomes were unaffected by kanamycin cast serious doubt on this proposal.

Davey *et al.* (1970) reported that cytosolic and mitochondrial ribosomes of rat liver and yeast were resistant to kanamycin. This work was subject to several flaws. Their conclusion that cytosolic ribosomes are unaffected by kanamycin is unsustainable because their assay efficiently detected only inhibition of translation, and not mistranslation, which Singh *et al.* (1979) found to be the primary effect of kanamycin. Secondly, inhibition of yeast and rat liver mitochondrial translation was observed, particularly with isolated mitochondria, however both systems were scored as resistant by comparison with the inhibition of yeast mitochondrial translation caused by erythromycin, lincomycin, neomycin

Table 3.1

Response of Cytosolic Ribosomes to Aminoglycoside Antibiotics.

Source	Kanamycin	G418 [c]	Hygromycin	Reference
S. cerevisiae	Sensitive [a]	N.D.	Sensitive	Singh *et al.* (1979)
Tetrahymena	Sensitive [a]	N.D.	N.D.	Palmer and Wilhelm (1978)
Frog	Sensitive [b]	N.D.	N.D.	Palmer and Wilhelm (1978)
Hamster	Sensitive [b]	N.D.	N.D.	Palmer and Wilhelm (1978)
Human cells	Sensitive [b]	N.D.	N.D.	Wilhelm *et al.* (1978a)
Wheat embryo	Sensitive [b]	N.D.	N.D.	Wilhelm *et al.* (1978b)
Simian Cells	N.D.	Sensitive	N.D.	Burke and Mogg (1985)

N.D. indicates that the response to this antibiotic was not determined.

[a] Yeast is most sensitive to kanamycin C *in vivo* , but cell free extracts are most sensitive to kanamycin B. *Tetrahymena* is most sensitive to kanamycin C *in vivo* and *in vitro* . Commercial preparations are a mixture of kanamycins A, B, and C.

[b] Tested in cell free extracts.

[c] This antibiotic is sometimes used in place of kanamycin when selecting with the NPT genes.

and paromomycin which occurred at 100 fold lower concentrations. Rat liver mitochondrial protein synthesis was reported also to be resistant to all the above antibiotics and to streptomycin. In contrast to this, Kroon and DeVries (1970) showed that mild swelling of rat liver mitochondria induced susceptibility to erythromycin and lincomycin, implying that membrane impermeability was the cause of the resistance observed by Davey *et al.* (1970). A similar mechanism has been proposed by Borst and Grivell (1971) to explain the observed resistance of yeast and rat liver mitochondrial protein synthesis to the other antibiotics.

In support of this, although Davey *et al.* (1970) reported that protein synthesis in isolated yeast mitochondria was resistant to streptomycin but not erythromycin, Scragg *et al.* (1979) showed that a protein synthesising fraction obtained from yeast mitochondria was inhibited by streptomycin almost as efficiently as with erythromycin, though kanamycin was not examined. Kurtz (1974) developed a similar submitochondrial fraction from chicken embryos and demonstrated that though neomycin was most effective, translation was inhibited by streptomycin and kanamycin, with the latter also inducing misreading. Both these antibiotics were more effective than erythromycin. Furthermore, Bunn *et al.* (1970) concluded that, *in vivo*, resistance of yeast mitochondrial protein synthesis to mikamycin and chloramphenicol in antibiotic resistant mutants was also attributable to membrane impermeability.

In plants, analysis of protein synthesis in isolated mitochondria and of mutations to antibiotic resistance suggests that plant mitochondria are relatively insensitive to a range of antibiotics similar to that claimed for rat liver mitochondria; this includes streptomycin (Maliga *et al.* 1975, Newton and Walbot 1985), lincomycin (Cseplo and Maliga 1982, Leaver 1975), erythromycin (Tassi *et al.* 1983, Boutry *et al.* 1984), and kanamycin (unpublished work of A.D. Liddel). A submitochondrial protein synthesising fraction is not available to examine the origin of resistance, though Manna and Brennicke (1985) have suggested, on the basis of sequence comparison with a resistant strain of *E. coli* , that erythromycin resistance may lie with the mitochondrial 26S rRNA of maize and *Oenothera* .

Taken together these results indicate that whenever investigated, protein synthesis in intact mitochondria *in vivo* or *in vitro* is resistant to kanamycin and a range of other antibiotics, however in each documented case, this resistance can be attributed to membrane impermeability rather than insensitivity of the ribosomes. Were this to be the case for tobacco, *nptII* expressed in the mitochondrion would probably be ineffective. There is no reason to believe that the enzyme could leave the mitochondria, but if confined within it would be isolated from the antibiotic and the sites of antibiotic sensitivity outside the mitochondria. In the absence of direct evidence to refute this possibility and to establish the effect of kanamycin in plant cells, it was decided that kanamycin could not form the basis of a selection system for mitochondrial transformation.

Even less is known about the effects of hygromycin on eukaryotic cells, so it was also excluded, leaving chloramphenicol as the only established alternative.

The D-threo-chloramphenicol isomer of chloramphenicol (hereafter referred to simply as chloramphenicol) has been shown in many systems to be an effective inhibitor of organellar translation, though it has not been demonstrated to inhibit cytoplasmic ribosomes (Table 3.2). It has been shown also to directly inhibit sulphate uptake, photophosphorylation, and mitochondrial respiration *in vivo* and *in vitro* (Ellis 1977). Inhibition of protein synthesis arises from its binding to the large ribosomal subunit and preventing peptide bond formation. The sensitivity of both chloroplast and mitochondrial ribosomes to chloramphenicol raises the possibility that *cat* expressed in one organelle would not provide sufficient resistance to allow adequate protein synthesis in the other.

Several observations suggested that in fact this may not be a problem. Firstly, in the investigations referred to above, access of chloramphenicol to the mitochondrial matrix was not restricted by the organelle membranes which seemed to present a barrier to many aminoglycoside antibiotics. Thus, given sufficient CAT activity, free diffusion of chloramphenicol could potentially allow its intracellular concentration to be effectively reduced. Secondly, a plant expressing CAT in only its chloroplasts was selectable on chloramphenicol (DeBlock *et al.* 1985); interestingly, this plant also expressed a cotransferred NPT gene but was not detectably resistant to kanamycin. Thirdly, callus tissue frequently lacks developed chloroplasts, and can be grown in the dark indicating that, under conditions used to culture and select transformants, the photosynthetic activity of chloroplasts at least can be dispensed with. Tassi *et al.* (1983) have taken this further by comparing the effects of chloramphenicol on tobacco callus with those of erythromycin. They showed that the chloroplast translation system specifically could be inhibited with erythromycin, leading to chlorosis and severe disruption of chloroplasts, but not to reduction in cytochrome oxidase activity or growth rates. Chloramphenicol also induced chlorosis, however it also caused marked reduction in cytochrome oxidase activity and growth rates, which was attributed to its additional effects on the mitochondria. Similarly, chloroplasts in tobacco callus are sensitive to streptomycin at below 500 µg/ml, becoming chlorotic and severely deformed; significant ultrastructural changes to mitochondria were not observed at concentrations below 1 mg/ml (Zamski and Umiel 1978, Umiel *et al.*1978). Growth continued at about half the normal rate on 500 µg/ml streptomycin, suggesting that there is not a stringent requirement for chloroplast encoded functions in tissue culture. If this is the case the degree of cross protection that must be afforded by CAT in the mitochondria is decreased. However, in the same study, chloroplast mutations to streptomycin resistance led to partial restoration of growth rates indicating that the cell is not entirely insensitive to chloroplast

Table 3.2

Response of Cytosolic and Organellar Ribosomes to Chloramphenicol.

Source	Cytosolic	Mitochondrial	Chloroplast	Reference
Yeast	Resistant	Sensitive		Davey *et al.* (1970)
Tetrahymena		Sensitive		Allen and Suyama(1972)
				Mager (1960)
Rat		Sensitive		Beattie *et al.* (1967)
Bovine		Sensitive		Kroon (1963)
Chick	Resistant	Sensitive		Kurtz (1974)
Wheat	Resistant[a]	Sensitive		Wilhelm *et al.* (1978a)
				Boutry *et al.* (1984)
Soybean	Resistant		Sensitive	Barraclough and Ellis (1979)
Barley	Resistant		Sensitive	Criddle and Dau (1970)
Sugar beet		Sensitive		Boutry *et al.* (1984)
Pisum	Resistant		Sensitive	Cashmore (1976)
Vicia	Resistant	Sensitive	Sensitive	Machold and Aurich (1972)
				Boutry *et al.* (1984)
N. tabacum		Sensitive		Boutry *et al.* (1984)
N. sylvestris		Sensitive	Sensitive	Tassi *et al.* (1983)
C. reinhardtii	Resistant		Sensitive	Iwanij et al. (1975)
Euglena	Resistant	Sensitive	Sensitive	Neuman and Partier (1973)

a *in vitro*

The response is indicated for each type of ribosome that was investigated in the references cited.

disruption.

Thus chloramphenicol was chosen as the most suitable selective agent, though the consequences of its action on chloroplast ribosomes, and the degree of cross protection between cellular compartments that could be expected were not clear. These were investigated during the course of the work (Chapter 6).

3.1.3 Source of the CAT Gene.

In previous studies, the chloramphenicol acetyltransferase gene (*cat*) from the bacterial transposable element Tn9 has been used as a selectable marker. This enzyme inactivates chloramphenicol by catalysing the transfer of acetyl groups from acetyl coenzyme A to the 3-hydroxyl group of chloramphenicol. Following an enzyme independent isomerisation step in which the acetyl group is transferred to the 1-hydroxyl group, the enzyme can perform a second acetylation at the first position.

Unfortunately the Tn9 gene encodes one of the arginine residues of CAT by a CGG codon (Marcoli *et al.* 1980). As mentioned in section 1.1, there is good circumstantial evidence that this codon may specify tryptophan in plant mitochondria, whereas it specifies arginine in the standard genetic code. If this gene was expressed in plant mitochondria, the arginine residue which is highly conserved at this position (Figure 3.1) may be substituted by tryptophan, and the enzymatic activity of the protein may be disrupted. The evidence for assigning CGG as a tryptophan codon, and its consequences for expression of *cat* in plant mitochondria are presented and discussed below.

Deviation from the Standard Genetic Code in the Mitochondrial Genetic Codes of other Organisms.

Several precedents exist for deviation from the standard genetic code such as that proposed for plant mitochondria. All other mitochondrial codes except that of *Chlamydomonas reinhardtii* have been shown to differ from the standard code; these differences are listed in Table 3.3.

Table 3.3

Code	Codon UGA	UAA	AUA	AGA	AGG	CUN
Standard	Stop	Stop	Ile	Arg	Arg	Leu
Plants	Stop	Stop	Ile	Arg	Arg	Leu
N. crassa	Trp	Stop	Ile	Arg	Arg	Leu
Trypanosomes	Trp	Stop	Ile	Arg	Arg	Leu
S. cerevisiae	Trp	Stop	Met	Arg	Arg	Thr
Insects	Trp	Stop	Met	Ser	Arg	Leu
Mammals	Trp	Stop	Met	Stop	Stop	Leu
Tetrahymena	Glu	Glu	Ile	Arg	Arg	Leu

The codons whose specification is found to vary in mitochondrial genetic codes are shown at the top of the table. Below these are listed the amino acids they specify in the standard code and that of the mitochondria of the organisms shown on the left. (Compiled from Grivell 1986, and Grivell 1983).

Do Plant Mitochondria Translate CGG codons as Tryptophan or Arginine ?

The proposition that CGG specifies tryptophan in plant mitochondrial genes was made and supported following comparisons of the predicted amino acid sequences of plant mitochondrial proteins with their homologues from other organisms. These revealed that highly conserved tryptophan residues occur in the homologous proteins at positions corresponding to CGG codons in plant mitochondrial genes (Fox and Leaver 1981, Hiesel and Brennicke 1983). There is still no direct evidence for this proposal, however there are now far more plant mitochondrial gene sequences on which to base the comparison than there were at the begining of this work. The CGG codon occurs in ten of the plant mitochondrial genes sequenced to date, and in total at 19 different positions. Table 3.4 lists the amino acid residues that occur at each of these 19 positions in the homologous proteins from a variety of other organisms. At ten of these positions CGG aligns with a tryptophan residue, all of which are highly conserved; of the 51 residues found at these positions that are not encoded by CGG , 50 are tryptophan. At 6 positions arginine is found in at least one species, though at only two positions is this residue conserved; only 16 of the 25 residues at these positions that are not encoded by CGG are arginine. At the remaining 3 positions neither arginine nor tryptophan occurs in any of the homologues but a variety of generally hydrophobic residues are found.

Table 3.4

Origin	Gene									
	coxI	*coxII*	*coxIII*	*cob*	*nad3*	*rps14*	*nad5*	*atpA*	*rps12*	*rps13*
Sorghum	T C	- - - - -	- -	- -	-	--	- -	--	- -	- -
Maize	T C	C C C T	- -	T C	C	--	- -	R	C C	R C
Wheat	T C	C C C T	- -	C C	C	-	- -	--	C C	R C
Oenothera	C C	C C C C	C C	C T	--	--	C C	R	- -	C C
Soybean	C C	C C C C	- -	- -	--	--	- -	--	- -	- -
Rice	- -	C C C T	- -	- -	--	--	- -	--	- -	- -
Broad Bean	- -	- - - - -	- -	C T	--	C	- -	--	- -	- -
Pea	- -	C C C C	- -	- -	--	--	- -	C	- -	- -
Petunia	- -	- - - - -	- -	- -	C	-	- -	--	- -	- -
Tobacco	- -	- - - - -	- -	- -	--	--	- -	R	- -	R C
Yeast	W L	W W W W	W W	W V	--	--	- -	L	- -	- -
Aspergillus	W L	- - - - -	- -	W I	--	--	- -	--	- -	- -
Neurospora	W L	- - - - -	W W	W I	--	--	R A	--	- -	- -
B. taurus	- -	W W W	- -	W A	W	--	- -	M	- -	- -
Human	W Y	- - - - -	W W	W L	W	--	- -	--	- -	- -
Mouse	W F	- - - - -	- -	W I	W	--	R T	--	- -	- -
Rat	- -	- - - - -	- -	W I	--	--	- -	--	- -	- -
X. laevis	- -	- - - - -	- -	- -	W	--	- -	--	- -	- -
Drosophila	W L	W W	- -	- -	--	--	- -	--	- -	- -
Trypanosome	W I	- - - - -	- -	- -	--	--	- -	--	- -	- -
C. reinhardtii	W L	- - - - -	- -	- -	--	--	A F	-	- -	- -
Euglena Chl.	- -	- - - - -	- -	- -	-	--	- -	--	K R	- -
LiverwortChl.	- -	- - - - -	- -	- -	W	W	- -	--	E R	- -
Tobacco Chl.	- -	- - - - -	- -	- -	W	W	G G	L	R R	- -
SpinnachChl.	- -	- - - - -	- -	- -	W	--	- -	--	- -	- -
Pea Chl.	- -	- - - - -	- -	- -	W	--	- -	--	- -	- -
Maize Chl.	- -	- - - - -	- -	- -	T	--	- -	--	- -	- -
E. coli	- -	- - - - -	- -	- -	--	W	- -	--	V R	R R
Bacillus sp.	- -	- - - - -	- -	- -	--	--	- -	--	V R	- -

Each column in the table lists the amino acids that occur at one of the 19 positions where CGG codons have been found in plant mitochondrial genes. These genes are listed at the top of each column. In the top half of the table, the presence of CGG is indicated by C, and the other codons for tryptophan and arginine by T and R respectively. In the lower half, the amino acids present at these positions in some of the homologous proteins from other species are indicated in standard single letter notation. Chl. denotes a chloroplast gene. Dashes indicate that the sequence is not available, or was not included in the published comparison. A space has been left if the corresponding residue is not clearly assignable. The table was compiled from the references given by Lonsdale (1988).

As CGG codons align with conserved residues of both amino acids, this simple comparison does not allow its proper designation to be decided. However, based on this comparison, either CGG codons are translated as tryptophan residues, or, if they are translated as arginine, massive substitution of tryptophan by arginine has occurred during the evolution of plant mitochondrial proteins.

If the former is true, and CGG codons are translated as tryptophan, such codons at positions of conserved arginine residues represent substitution of arginine by tryptophan. One might therefore expect arginine codons similarly to have been replaced by the more abundant (Table 7.3) tryptophan codon TGG. However, this is not observed in any of the mitochondrial genes sequenced to date; only one TGG codon in plant mitochondrial genes aligns with an arginine residue in a homologous protein, and this arginine residue occurs in only the *Neurospora crassa* homologue.

If the latter is true, it appears that conserved tryptophan residues have frequently been replaced in plant mitochondrial proteins, by arginine residues that are encoded by CGG. However there are six different codons in the standard genetic code that specify arginine, so one might expect that some generally conserved tryptophan residues would also have been replaced in plant mitochondrial proteins by arginine residues encoded by one of the other five available codons. This is not observed. In all the plant mitochondrial genes analysed to date these alternative arginine codons align with a tryptophan residue at only two positions, both of which are in the highly divergent carboxy terminus of the respective proteins; one of these tryptophan residues is in ATP A of yeast, and the other is in COXI of *Neurospora* .

Thus the evidence from such sequence comparisons does not allow an amino acid to be confidently assigned to the CGG codon, though as it aligns with conserved tryptophan residues most frequently this it is perhaps most likely to specify this amino acid.

The Consequences of Substituting Tryptophan and Arginine Codons.

If CGG is translated as arginine in higher plant mitochondria as in the standard genetic code, it follows that several conserved tryptophan residues in mitochondrial proteins have been substituted by arginine in the higher plant homologues. However, if it is translated as tryptophan, the highly conserved arginine residue encoded by CGG in the CAT gene of Tn*9* would be replaced with tryptophan by the plant mitochondrial translation machinery. Substitution of tryptophan and arginine can potentially cause serious disruption of protein function, making it difficult to accept the former, and raising concern about the

latter. Table 3.5 shows that during evolution the substitution frequency for these two amino acids in homologous proteins is low. Tryptophan is hydrophobic and arginine is highly positively charged so they are unlikely to perform similar catalytic functions, but more seriously the protein's tertiary structure, which frequently is dependent upon hydrophobic interactions, is likely to be disrupted. These two residues coincide 0.3 times as frequently as expected by chance, implying that this substitution is unfavourable and therefore suppressed. Tryptophan is clearly the best conserved of the amino acids; it is found to occur at the same position in homologous proteins 41.4 times more frequently than expected, suggesting that its function is not easily substituted. Though tryptophan is almost the least acceptable alternative to arginine, many amino acids are less acceptable alternatives to tryptophan than is arginine. This may be due to the large size of the R-groups of these two amino acids, a factor considered important in packing of residues in the protein. The energy required to introduce either of these into an unfavourable physical environment is similar to the total energy of folding of many proteins (Schultz and Schirmer 1985). It would be remarkable if a hydrophobic protein such as COXII, which has four of its eight potential tryptophan residues encoded by CGG, was to have arginine residues substituted at these positions.

The physical environment and function of the CGG encoded arginine residue of CAT, is unknown so the effects of introducing tryptophan here are unclear. Therefore, whilst the strong possibility remains that this codon specifies tryptophan in plant mitochondria, the CAT gene of Tn*9* which has been used previously as a selectable marker is not ideal. Instead, a CAT gene from *Proteus mirabilis* was chosen (Charles *et al.* 1985a and b). This is a chromosomal gene encoding a product that has over 80% amino acid identity with the Tn*9* CAT variant, (Figure 3.1), but does not contain CGG codons. This and the fact that it shows a bias that is shared with plant mitochondrial genes, for codons ending in adenosine or thymidine (Dawson *et al.* 1986a and b) suggest that the *P. mirabilis* CAT variant is well suited to expression in plant mitochondria.

The potential problem associated with selectable marker genes that contain CGG codons extends to the NPT genes as all the variants available contain between three and six CGG codons (Oka *et al.* 1981, Beck et *al.* 1982, Trieu-Cuot and Courvalin 1983).

Table 3.5

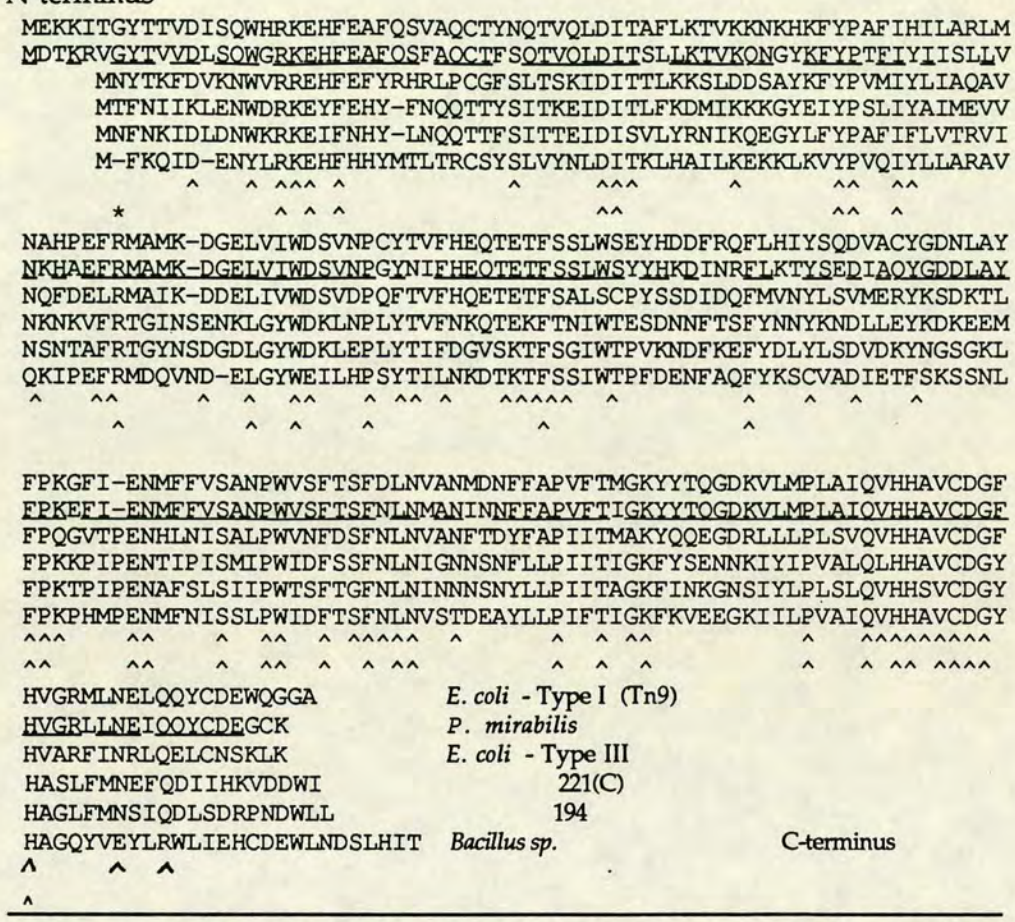
Matrix of Relative Substitution Frequencies at an Evolutionary Distance of 256 PAM.

	G	P	D	E	A	N	Q	S	T	K	R	H	V	I	M	C	L	F	Y	W
G	29																			
P	12	14																		
D	11	10	23																	
E	11	11	19	20																
A	13	14	12	12	14															
N	11	10	14	12	12	17														
Q	10	11	14	14	11	12	21													
S	12	11	12	11	13	13	11	13												
T	10	10	10	10	12	12	11	13	18											
K	7	8	10	10	9	12	11	10	9	24										
R	4	4	7	7	5	10	13	7	6	22	**75**									
H	6	6	9	8	8	13	12	9	9	11	20	59								
V	7	8	7	8	10	8	9	9	11	8	5	6	22							
I	6	6	6	7	8	7	8	8	10	7	5	7	21	25						
M	5	6	6	6	8	7	8	8	9	8	10	6	16	16	26					
C	6	4	5	5	7	6	5	11	9	4	2	3	11	9	12	166				
L	4	4	5	6	6	5	6	6	7	6	4	6	15	18	21	5	40			
F	2	2	2	3	4	3	3	4	5	3	4	7	7	11	11	2	12	70		
Y	1	1	1	1	2	2	2	3	2	3	3	7	3	6	6	1	6	66	137	
W	1	1	1	1	1	2	2	2	2	2	3	15	2	4	4	1	3	41	46	**414**

The twenty amino acids are represented by the standard single letter notation along the axes of the matrix. Each value is the probability calculated from observed substitution frequencies that a pair of amino acids will occur at a given position in homologous proteins divided by the probability that it will occur by chance. The ratios are then multiplied by 10. Clearly the frequency of substitution of one amino acid for another depends upon the time since the two proteins separated from their common ancestor, the relative mutation rates of their genes, the likelihood that a mutation will convert a codon for one amino acid into a codon for the other, and finally upon the acceptability of the substitution for proper protein function. All but the last of these influences can be evaluated and used to calculate the probability of each substitution after any particular degree of divergence has occurred, assuming all amino acids to be functionally equivalent. Deviation from the prediction can be attributed to selection for or against a particular substitution, most probably on the basis of their functional equivalence. These values have been calculated for an evolutionary distance of 256 PAM, which means in effect that the proteins compared have been separated for such time that there are 256 point mutations per 300 bp (100 codons) between their respective genes. The substitution frequencies for arginine and for tryptophan are underlined, and the most important values are in bold face. All data is taken from Schultz and Schirmer (1985).

Figure 3.1

Protein Sequences Predicted from Several Bacterial CAT Genes.



The predicted amino acid sequences derived from six different CAT genes are shown in the standard single letter notation. The residues that are identical in the Tn*9* and the *P. mirabilis* variants are underlined below the latter sequence. Residues that occur in five of the six proteins are indicated by an arrowhead below the *Bacillus* sequence, and those that are conserved in them all by two arrowheads. The arginine residue encoded by CGG in the Tn*9* sequence is marked by an asterisk. The sequence of the Tn*9* protein is taken from Marcoli *et al.* (1979), that of *P. mirabilis* from Charles *et al.* (1985b) and this work, and the remainder from Dr. I. Murray, University of Liecester.

3.1.4 Expression of the Selectable Marker Gene.

Initially, expression of the selectable marker requires that a promoter recognised by tobacco mitochondrial RNA polymerase is provided to initiate transcription. It is still not clear what sequences are necessary for promoter activity in plant mitochondria as there is only circumstantial evidence available, however transcription of yeast and mammalian mitochondrial genes is better understood.

3.1.4.1 Promoters in Yeast Mitochondrial DNA.

At least 19 different transcript initiation sites exist, and appear to be spread randomly through the 80 kb genome (Leven *et al.* 1981). Some transcripts encode a single product; others encode more than one but such grouping does not appear to reflect their related function as it does in the polycistronic messages of prokaryotes (Miller and Reznikoff 1978); conversely some genes are transcribed from more than one promoter.

The nucleotide at which transcription initiated, and the first eight bases upstream of this nucleotide were found to be very similar in all the initiation sites analysed (Osinga *et al.* 1984b). The initiation sites for the 21S rRNA, 18S rRNA, ATP9 and COXI genes and four sites in the replication origin were all found to be at the last base in the sequence 5' ATATAAGTA 3'. The corresponding sequence for four tRNA genes that were also analysed all varied from this at a single position. Edwards *et al.* (1982) showed that *in vitro*, partially purified yeast mitochondrial RNA polymerase could initiate transcription at these sequences in cloned fragments of mitochondrial DNA.

Using this system to analyse the effects of deletions introduced *in vitro* into the 14S rRNA promoter region, Biswas *et al.* (1985) concluded that not more than nucleotides -10 to +2 relative to the initiation site are necessary for full transcriptional activity, but that deletions into the conserved region progressively reduce activity (the adenosine at which transcription initiates is designated +1, and numbering continues downstream from there; the thymidine immediately upstream is designated -1 and numbering continues upstream from there). Similar conclusions were drawn by Tabak *et al.* (1983) who introduced point mutations into and around the promoter region, though a double mutation at positions +10 and +13 impeded promoter activity. It is surprising that the determinants of promoter function reside almost entirely within this highly conserved region as the relative strength of different promoters is found to differ

widely. The difference in activity between promoters may be due to the nucleotides at positions -8 and +2 that do vary (Mueller and Getz 1986a), or to sequences outside this region that reduce activity in the weaker promoters as the 14S rRNA gene promoter, on which the mutation analysis was performed, is among the strongest (Biswas *et al.* 1985). A similar conserved promoter sequence is found at transcription initiation sites of the distantly related yeast *Kluyveromyces lactis* (Osinga *et al.* 1984).

Schinkel *et al.* (1987) have shown that the yeast mitochondrial RNA polymerase is an oligo-dimer. The 'core' subunit has polymerase activity and shows structural similarities to the RNA polymerases of bacteriophages T3 and T7 (Masters *et al.* 1987). The 'specificity' subunit has no endogenous polymerase activity, but confers preference for initiation of transcription at the conserved nonanucleotide consensus sequence.

3.1.4.2 Promoters in Vertebrate Mitochondrial DNA.

In human mitochondria, transcription initiates from two closely spaced divergent promoters to give full or subgenomic length transcripts from each strand of the genomic DNA molecule. These transcripts are then specifically cleaved to generate the final structural and messenger RNA species. The promoter of the light strand is also implicated in priming DNA replication (Chang and Clayton 1985). By analysing the effects of deletions and point mutations on transcription *in vitro* from these promoters, the minimal sequence showing promoter activity has been determined to consist of the sequence 5' GGGGTTT 3' for the heavy strand, and 5' GGCGGTTTT 3' for the light strand, with transcription initiating at one of the thymidine residues (Chang and Clayton 1984, Hixson and Clayton 1985). A single mutation of several introduced around -30 (relative to the start of transcription) reduced the transcription rate providing some evidence for the influence of flanking sequences. Fischer *et al.* (1987) have identified a transcription factor that binds immediately upstream of these two promoters and provides sequence specificity to the non-specific RNA polymerase. It appears to act in an orientation independent fashion, and its binding site extends into the -30 region. Thus the structure of the RNA polymerase and its interaction with the promoter appear to be similar in both yeast and vertebrate mitochondria despite distinct differences in their promoter sequences and in the way transcription of their mtDNA is organised. Also, in both systems, the DNA sequences that bind the polymerase complex are equivalent to those conserved sequences at which transcription initiates.

The human mtDNA promoter sequences are conserved in four hominid

species with varying stringency, (for example the sequence of the transcription factor binding site varies even between humans), but they do not appear to have been conserved in other vertebrates though their genomes are transcribed in similar fashion (Foran *et al.* 1988).

3.1.4.3 Transcription of Plant Mitochondrial Genes, and Promoters in Plant Mitochondrial DNA.

Our knowledge of transcription in plant mitochondria is limited to sequence and transcript analysis because an *in vitro* transcription system with which to analyse potential promoters is lacking. From the earliest analysis, transcription of plant mitochondrial genes was found to be complex, resembling that of yeast more closely than mammals. Northern blots probed with cloned sequences revealed that several genes generate complex arrays of transcripts, some of which are many times larger than required to encode the polypeptide. Such genes include *cob* in maize, *Oenothera* , and broad bean (Dawson *et al.* 1984, Schuster and Brennicke 1985, Wahleitner and Wolstenholme 1988b), *coxII* and *atpa* of maize (Fox and Leaver 1981, Isaac *et al.* 1985a), *atp9* of maize, tobacco and broad bean (Dewey *et al.* 1985, Bland *et al.* 1986, Wahleitner and Wolstenholme 1988b), and *atp6* of tobacco (Bland *et al.* 1987). Though some of the apparent complexity has been shown to arise from individual gene probes hybridising to transcripts from more than one gene (for example *atp9* and *coxI* in maize, and *atp6* and ORF S13 in tobacco), many of the labelled bands appear to represent multiple transcripts for the same region. These observations imply that multiple initiation, termination and processing events could be involved in transcription of many mitochondrial genes.

Some of the transcripts for the genes known to contain introns represent unspliced precursors and excised intron sequences, which contribute to the apparent complexity (Fox and Leaver 1981, Bonen *et al.* 1984, Wissinger *et al.* 1988). Such post-transcriptional processing events, and those that generate the mature 18S and 26S rRNAs in maize (Mulligan *et al.* 1988b) are the only established examples of a process that is frequently supposed to combine with transcription initiation in generating these complex Northern hybridisation patterns. Similar processes may be involved in generating the mature rRNAs in other plant species, and *nad5* mRNA from precursors cotranscribed with the 18S and 5S rRNAs of *Oenothera* (Wissinger *et al.* 1988).

However, some plant mitochondrial genes have relatively simple transcription patterns, and the 5' ends of their transcripts have been mapped on cloned fragments of mtDNA by primer extension or S1 protection analysis. These

Figure 3.2 DNA Sequence Homologies at Plant Mitochondrial Transcript Termini.

The upper part of this table compares the sequences in plant mitochondrial DNA around the sites to which the 5' termini of several transcripts have been mapped. These sites are indicated by asterisks, and have been used to align all the sequences except the one from *rrn18* of *Oenothera* . The sequences represent the coding strand of the DNA and are presented with their 5' ends to the left. They are arranged in two 15 bp blocks which are contiguous in mtDNA; the conserved bases which immediately flank the transcript termini form one block, and the 15 bp immediately upstream of these form the other. At each position, bases that occur in at least 50 % of sequences were used to derive the consensus for each block, and these are shown; the percentage of sequences that contain the consensus base is shown (to the nearest 10 %) below each position.

The 15 bp block surrounding the transcript termini is clearly similar in all these sequences. Theoretically, such similarity could reflect either a general sequence homology in plant mtDNA, or selection for sequences that fulfil particular functions at specific locations within regions of mtDNA that are generally dissimilar. Observation suggests that the former is not the case; in general, mtDNA upstream of plant mitochondrial genes shows minimal sequence homology, and this is reflected by the relatively poor consensus that can be derived from the 15 bp immediately upstream of the conserved block. Thus selection for a specific sequence appears to have occurred, and to have been confined to the sequence immediately flanking the transcript termini suggesting that the consensus sequence in this region may function in generating these termini.

Never-the-less, it is also possible that much of the sequence of the putative consensus (5' AAATNTCATAAGTGA 3') is frequently repeated in mtDNA, in which case much of it may be of little significance in determining the location of transcript termini. To investigate this, the putative consensus has been aligned with both 15 bp sequence blocks, and in each sequence the number of bases that match the consensus (written in upper case in the table) were determined; these numbers are presented in the column headed #/15. None of the upstream sequences have as many matches as those downstream, and averages of 5.2 and 13 matches per 15 bp can be calculated for the upstream and downstream sequence blocks respectively; 4.5 matches are expected by chance (if all bases were equally represented in mtDNA) with each additional one having 25 % probability. Thus the consensus is preferentially represented around transcript termini. A,C,G and T stand for one each of the four deoxyribonucleotides, and N for any one of the four.

At the bottom of the figure, the sequences surrounding two transcript termini that are clearly similar to related to those above but which require gaps to be introduce to produce the correct alignment are shown. Bases that match the consensus are in upper case, and those that must be removed for correct alignment are in subscript.

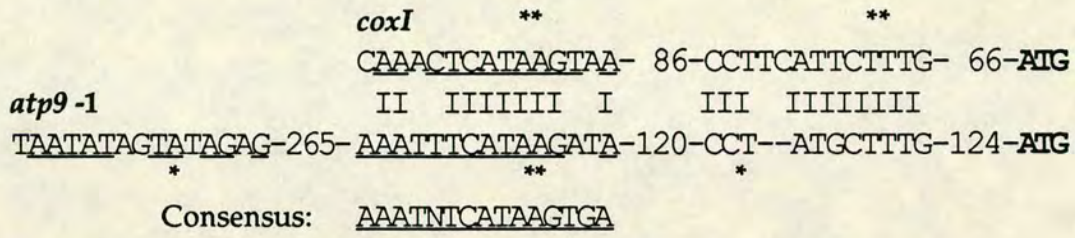
Figure 3.2 DNA Sequence Homologies at Plant Mitochondrial Transcript Termini.

Origin	Gene	Upstream Sequence	Sequence Around 5' Termini	#/15
			* *	
Oenothera	*atp6*	t t A g A g a t c g g c t g A	t A t T A T C A T A A G T G A	3 13
Oenothera	*atpA*	t t c T A a a g a A A G T t g	A t A a A T C A T A A G a G A	6 12
Oenothera	*coxI*	A g t c T T C c T g t t c a A	c A A T T g C g T A A G T G A	6 12
Oenothera	*coxII*	t t t a C c t c T A A c T a A	A A A T C T C g T A t G T G A	6 13
Oenothera	18S*rrn*	c A A a A a a c a A g a T t g	A A A T G T C A T A A G T*G A	5 15
Pea	*coxII*	A A t c C c t t a t t c T a t	A A A T T T a c T A A G a G A	4 12
Pea	*coxII*	A t t T A c t A a g A G a a g	A A A T C a C g T A A G T G A	6 14
Maize	*coxI*	t A c T T T t g c A c c g a A	g A A a C T C A T A A G T a A	6 12
Petunia	*atp9*- 1	t t c T T c t t a c A a a a g	A A A T T T C A T A A G a t A	3 13
Petunia	*atp9* -2	t t t T C a t c c A A c T a t	c A A T C T C g T A A G a G A	5 12
Sugar Beet	1.3 kb Mc	A A c T C T g t a t t t T G c	A A A T A c C A T A A G T G A	7 14
Sugar Beet	1.4 kb Mc	t A g g A g C t a A t t a G c	t A A a A T C A T A A G T G A	5 13
Sugar Beet	1.6 kb Mc	t t t T C T t g T t t t T t g	A A A T A T C g T A A G T G A	5 14
Consensus		T T N T N N T N A N N N T A N	A A A T N T C A T A A G T G A	
		60 50 -- 50 -- -- 50 -- 50 -- -- -- 50 50 --	60 90 90 80 -- 80 90 50 100 100 90 100 70 90 100	
Petunia	*atp9* -1		t A A T A T a G T A t A G T a t	
Maize	S-Plasmid		c A A T C T a C G T A a A G A t A	

termini usually map to within 600 bp of the translation initiation codon. Significant homology was detected in the DNA sequences at many of these positions, allowing consensus sequences for transcript 5' termini to be proposed. In Figure 3.2 I have used these homologous sequences to derive a consensus sequence similar to that proposed by Hiesel and Brennicke (1985). This consensus sequence occurs in the DNA at the 5' termini of messenger RNAs, ribosomal RNA precursors, and transcripts of unknown function from minicircles or 'plasmids'. Such transcribed minicircles must possess promoters within their relatively short sequence, and thus were useful in showing that the consensus sequence is not only well conserved, but also of predictive value. Thomas (1986) reported that the 1.6 and 1.3 kb minicircles of sugarbeet were transcribed. When their sequence was simply compared with the consensus, the region with greatest homology in each plasmid exactly predicted the nucleotide to which the 5' termini of the transcripts were subsequently found to map (C.Thomas, personal communication). By analogy with the conserved sequences at the 5' termini of the yeast and vertebrate mitochondrial transcripts, this consensus sequence has been regarded as a likely candidate for a plant mitochondrial promoter because it occurs at the 5' terminus of the longest detectable transcript from several plant mitochondrial genes. In fact, sequence similarity between the putative plant mitochondrial promoter, 5'AAATNTCATAAGTGA 3' and the yeast counterpart 5' ATATAAGTA 3' has been noted (Isaac *et al.* 1985b); however, the transcript termini map to different points within these sequences, and the significance of the similarity is not clear.

Some sequences, not included in Figure 3.2, which occur in mtDNA around the 5' termini of other transcripts do not fit this consensus. In general these other sequences show very limited similarity to each other. They are often located at the termini of transcripts shorter than those used to derive the consensus sequence above, and some have been regarded as potential processing sites (Isaac *et al.* 1985b, Young *et al.* 1986). Examples of genes with transcripts of each sort are *coxI* of maize, and *atp9*- 1 of *Petunia hybrida* (Figure 3.3). However, there are exceptions to these generalisations. For example the longest transcript of the Sorghum COXI gene and one of those for *atp9* -2 of *Petunia* map to regions that do not show homology to the consensus sequence or the putative processing signals. In addition, the longest detected transcript of the latter gene maps to a sequence that is homologous to the putative processing signal of the maize COXI and *P. hybrida* ATP9-1 genes (Rothenberg and Hanson 1987b).

Figure 3.3
Sequences at Termini of Transcripts of *coxI* in Maize and *atp9* -1 in *Petunia*.



The sequences in mtDNA surrounding the transcript 5' termini of these two genes are drawn to show their similar organisation. *coxI* generates two transcripts, and *atp9* -1 generates three. For each gene, the coding strand of mtDNA is shown with its 5' end to the left. At the extreme right end ATG indicates the initiation codon of each gene. The numbers between the sequences indicate the number of nucleotides that separate them. Asterisks above the *coxI* sequence and below the *atp9* -1 sequence indicate the sites at which the 5' termini of the transcripts have been mapped. The shortest transcripts map to sequences that do not resemble the putative promoter consensus sequence, however they are similar to each other as indicated by the vertical lines between them. It has been proposed that transcripts are processed at these sites. The significance of the relatively poor sequence homology at these sites is unclear; this portion of the sequence upstream of *atp9*-1 is part of a much larger conserved sequence block found upstream of several plant mitochondrial genes though not *coxI* of maize (Bland *et al.* 1987, discussed in the text below). The 5' termini of the longer transcripts map to sites with significant similarity to each other and to the putative promoter consensus. The nucleotides that match the consensus are underlined. The longest transcript of *atp9* -1 maps to a sequence resembling the consensus, though poorly. Modified from Young *et al.* (1986).

The Consensus Sequence: Promoter or Processing Site?

It seems beyond reasonable doubt that the consensus sequence identified above is involved in generating transcript 5' termini. However, initially there was only circumstantial evidence to support the proposition that transcription initiates at this sequence in mtDNA rather than processing occurring at this sequence in longer primary transcripts. In the absence of an *in vitro* transcription system or of a mitochondrial transformation system, the only way to distinguish a primary transcript from a product of processing is to use the capping reaction catalysed by guanylyl transferase. This enzyme normally modifies the 5' terminal nucleotide of nuclear encoded mRNA molecules by catalysing the addition of a guanidine cap via a triple phosphodiester link between the 5' carbons of the two nucleotides. The reaction requires the RNA molecule to have a di- or triphosphorylated terminus. The initial nucleotide incorporated into a message retains its triphosphate, but subsequent nucleotides lose two phosphates upon polymerisation. Thus, 5' termini generated by subsequent processing events are, at most, monophosphorylated. The 5' termini of plant mitochondrial primary transcripts appear not to be modified leaving them susceptible to the capping reaction, and allowing primary transcripts to be distinguished from processing products. The reaction is followed by using ^{32}P labelled GTP as a substrate for guanylyl transferase.

The few capping studies that have been performed with plant mitochondrial transcripts have served to determine genuine initiation sites, but have confused rather than clarified the nature of the sequences that determine promoter activity. Firstly, both transcripts of the maize COXI gene were found to be cappable though only the longer one maps to a region with homology to the consensus sequence (Dr. W. Hauswirth, University of Florida, Gainsville, personal communication). Mulligan *et al.* (1988b) observed that the maize 26S rRNA is transcribed as a cappable precursor that is about 180 nucleotides longer than the non-cappable mature molecule to which it is subsequently processed. Similarly, the 18S and 5 S rRNA molecules are cotranscribed as a cappable precursor that is subsequently cleaved. Southern blots of cloned maize mtDNA have been hybridised with capped, and hence radioactive, mRNA and subsequently treated with ribonuclease, revealing that both *cob* and *atp9* have multiple initiation sites for primary transcripts (Mulligan *et al.* 1988a). The same authors used primer extension and S1 protection to determine the number and location of 5' termini of transcripts from *coxIII* and *atp9* . Two sites were found for the former and at least six for the latter, all within 600 bp of the coding region. Surprisingly, every one of these sites appeared to generate cappable transcripts, but their homology to the consensus sequence derived above and to each other was poor (Figure 3.4).

These observations suggested that the DNA sequences around many mapped

transcript termini are sites for initiation of transcription. However, recently, Kennel and Pring (1989) have reported capping experiments with transcripts of *atp6* and its derivative, the T-*urf13* in the T-type maize cytoplasm. These two loci generate a large number of transcripts extending several hundred nucleotides upstream. The 5' termini of many of them have been mapped on cloned mtDNA fragments, and those of transcripts from both genes map to similar positions. In contrast to the previous capping studies, only two transcripts of each gene were found to be cappable. Their 5' termini mapped close to each other (about 620 bp upstream of *atp6*, and about 245 bp upstream of T-*urf13* and close to a sequence with some similarity to those sequences used to derive the consensus in Figure 3.2 above (the underlined nucleotides in the sequence 5' CATTTTCATA-GAGAAAGATGTTCTG 3', the termini are in bold face). Although a gap must be introduced for maximum similarity, even without this modification, this sequence is a closer match to the consensus than that near any other *atp6* or T-*urf13* transcript terminus. This finding appears to support the original view that multiple initiation and processing events combine to generate plant mitochondrial transcript termini. However, the cappable transcripts were not the longest detected, and limited similarity to the consensus can be found in the mtDNA sequences near some of the other transcript termini, emphasising that the origin of plant mitochondrial transcripts is still not properly understood.

All these observations provide little insight into the sequences necessary for promoter activity, their location relative to the initiation point, or the significance of the homologies that were found at the transcript termini. As mentioned in section 1.3.3, Mulligan *et al.* (1988a) proposed that nearly all transcripts may originate by genuine initiation of transcription. In such a case, plant mitochondrial RNA polymerase may interact with DNA relatively non-specifically, or the sequences it recognises may be dispersed or lie at some distance from the site of transcription initiation, making them difficult to identify. In contrast, the results of Kennel and Pring (1989) imply that only a minority of transcripts arise by initiation, and provide tentative support for the involvement of the consensus sequence identified previously.

Figure 3. 4

DNA Sequences Surrounding the Termini of Cappable Maize Transcripts.

Gene	Sequence Surrounding Transcript Termini	Position (nucleotides)
coxIII	GATGAGAAATGACGTATCTTACGTATCGAAT	-455
	GATGAGAATTGACGTATTCAAAGTGGAAA	-315
rrn18	AATTGACATAGATAAATCTTTATC	-232
rrn26	AAAATCGTATAAAAATCAAGC	-184
atp9	AACCATTTAACAGTTCAAACC	-495
	CTAGGTTATAGCTTAACCGAC	-418
	GTTATTTATTCAAATTGGAAA	-288
	GGAAATAGCTAGAACTCCTGC	-272
	GCAGGCTTGCTGTACTGAATC	-251
	ACCTGTCAAGCCCAAGATAAG	-218
Consensus	NNANNTTNNTNAAANTNNAAC	
	--5--65--6-556-7--856	

The nucleotide to which the the transcript termini were mapped was used to align the sequences, and is shown in bold face. The position of this nucleotide relative to the start of translation or the 5' terminus of the mature rRNA is given in the second column. Sequences that are homologous to each other are either underlined or overlined by broken lines. A poor consensus can be derived from bases occurring in at least half the sequences, and is shown below the list; the number of sequences that contain the consensus base is shown at the bottom. The consensus may in part reflect the base composition of these regions, which is 65% A and T. No other conserved sequences were observed in the flanking regions in a computer aided search (Mulligan *et al.* (1988a), from which the data is taken.)

3.1.4.4 Choice of Promoter for the Selectable Marker.

When this work was begun, many of the observations described above were not published. Transcripts had been mapped upstream of *coxI* from maize (Isaac *et al.* 1985b), *coxII* from *Pisum* and *Oenothera* (Moon *et al.* 1985, Hiesel and Brennicke 1985), and on a sugar beet minicircular DNA (Hansen and Marker 1984).

It was of interest to determine whether plant mitochondrial promoters resembled those of other systems. The sequences around plant mitochondrial transcript 5' termini were surveyed for the conserved sequence elements that are responsible for initiating transcription of bacterial, chloroplast, and nuclear mRNA (Siebenlist *et al.* 1980, Gruissem and Zurawski 1985, Murphy*et al.* 1989, Joshi 1987). Some such sequences could be found; for example, the two transcripts of *coxII* in *Pisum* contain either a TATA or Pribnow box at 25 and 10 bp respectively upstream of their 5' termini, and *coxI* from S but not N cytoplasms of maize has a TATA box at about 25 bp upstream of its longest transcript. Similarities between the sequences surrounding plant and yeast mitochondrial transcript 5' termini have already been mentioned. There were however insufficient grounds to assume that plant mitochondrial promoters were equivalent to those of any other system. The two important consequences of this were that bacterial and nuclear promoters would probably be ineffective for transformation of mitochondria so necessitating the use of a genuine plant mitochondrial sequence, and that doing this may well provide the desired specificity to expression of the selectable gene.

The transcript termini that had been determined, and the corresponding flanking sequences that were initially available for use in the transformation vectors are listed above. All these apart from the shorter of the two transcripts from *coxI* mapped to sequences that showed homology to each other, and to the subsequently derived consensus (Figure 3.2). Because these transcripts were the largest detected, it was proposed that they arose by genuine initiation of transcription. The subsequent capping experiments described above using the longer transcript of *coxI* have supported this proposal. The shorter transcript of *coxI* terminated adjacent to a potential hairpin structure located 69 bp upstream of the initiation codon. A series of less abundant transcripts mapped to several of the nucleotides surrounding the terminus of the major transcript (Isaac *et al.* 1985b, and Valerie P. Jones 1985 Ph.D. Thesis University of Edinburgh). These features were interpreted as evidence for processing of the primary transcript, though it now appears that this transcript also is cappable.

The maize COXI Gene Promoter.

The putative promoter of the maize COXI gene was selected for use in the transformation vectors for the following reason. Though there was reason to believe that many of the sequences identified at the transcript termini constituted at least a component of a promoter, there was little to infer what additional, unrecognised, sequence may be required for its activity. However, analysis of the maize COXI gene in N and S cytoplasms suggested that the putative promoter sequence identified above may be sufficient (Isaac *et al.* 1985b). The sequence upstream of this gene in N and S cytoplasms is identical until 17 bp beyond the 5' terminus of their longest transcript. At this point in the N-type variant, there begins a 20 bp sequence that is homologous to the terminal nucleotides of the terminal inverted repeats (S-TIR) of the two S plasmids that characterise the S cytoplasm (Figure 3.5). Obviously, this 20 bp sequence is also homologous to the portion of the S-TIR sequence that normally exists in the N cytoplasm adjacent to the 5.27 kb repeat (see section 1.3.1). In the S cytoplasm, a recombination event appears to have occurred between this 20 bp sequence and one of its homologues. Subsequent recombinations between these homologues have led to the region from 17 bp beyond the terminus of the longest transcript being replaced by a complex set of sequences variously derived from each end of the two S plasmids and from a part of the region that flanks the 5.27 kb repeat in N cytoplasms (for example see Figure 3.6). The consequence of these rearrangements is that sequences from 17 bp upstream of *coxI* in S cytoplasms show only random homology to those in N cytoplasms. Despite this, when examined by Northern blotting, these replacements appear to have left the transcription of the gene totally unaffected (Isaac *et al.* 1985b). The simplest interpretation of this result is that all sequences affecting transcription of *coxI* reside downstream of the rearrangement point, and perhaps, by analogy with the short promoter sequences of yeast and human mitochondria, to within the conserved consensus. The possibility that upstream promoter elements have been fortuitously provided by some of the replacement sequences, or that such elements exist within or 3' of the coding region cannot be excluded. Never-the-less, the maize *coxI* promoter was the most extensively characterised, and was chosen to express *cat* in chimaeric transformation vectors.

The Promoter from an ATP9 Gene in *Petunia hybrida* .

Although there is no evidence that the sequence determinants of mitochondrial promoter activity vary in different plant species, promoters from

distantly related species may not be interchangeable. The *coxI* promoter described above originates from *Zea mays* , a monocot, whereas *N. tabacum* , a dicot, is the species of choice for transformation. Unfortunately, although mitochondrial genes have been isolated from the latter, their promoters have not yet been identified. However, Young *et al.* (1986) have characterised the promoter region of *atp9*-1 of *Petunia hybrida.* Of the species for which mitochondrial promoters have been identified, *P. hybrida* is the most closely related to *N. tabacum* , and, as described in section 5.2.1 and Figure 5.1, the ATP9 genes in these two species are highly homologous. Therefore the promoter of *atp9*-1 was chosen as it is likely to resemble tobacco mitochondrial promoters most closely.

The ATP9-1 gene was shown to generate three detectable transcripts that accumulate in a ratio of 100:5:1 with increasing size (Young *et al.* 1986). The 5' ends of the two longest transcripts mapped 536 and 256 bp upstream of the coding sequence at sites resembling the putative promoter consensus, though the resemblance to the consensus of the sequence at -536 bp was weaker than that at -256 bp (Figure 3.3). DNA sequence around the 5' terminus of the shortest transcript, which mapped 124 bp upstream of the coding sequence, showed little similarity to the promoter consensus. Young *et al.* (1986) noted that the sequence around this transcript terminus was similar to that around the shortest transcript of *coxI* in maize, and proposed that each sequence may be involved in transcript processing. Perhaps of greater significance is that examination of the sequence upstream of *atp9*-1 reveals that its shortest transcript maps immediately downstream of a conserved 30 bp sequence that has been found upstream of several other plant mitochondrial genes (Figure 5.1, and Bland *et al.* 1986, 1987). The latter authors suggested that this sequence was involved in gene expression, and a role in transcript processing now appears likely in view of the results of Mulligan *et al.* (1988b) and Kennel and Pring (1989). They showed that the 5' termini of the shortest transcripts of *atp6* , T-*urf13* , and *rrn26* all map within or adjacent to versions of this conserved sequence (Figure 5.1) and that all three transcript termini are generated by processing of larger precursor RNA molecules. It is therefore probable that the shortest transcript of *atp9* -1 in *P. hybrida* is also generated by RNA processing.

A second ATP9 gene, *atp9*-2, exists in *P. hybrida* mitochondria, but its transcripts are three times less abundant than those of *atp9* -1 (Rothenberg and Hanson 1987b).

3.1.4.5 The 3' Termini of Plant Mitochondrial Transcripts.

Transcripts of *atp9* -1 in *P. hybrida* terminate 95 nucleotides downstream of

the termination codon (Rothenberg and Hanson 1987b), and those of *coxI* in maize are suggested to terminate about 450 nucleotides downstream of the termination codon (Schuster *et al.* 1986). Generation of the 3' termini of plant mitochondrial transcripts is not well characterised, but is addressed in Chapter 5.

3.1.4.6 Initiation of Translation.

Initiation of translation of plant mitochondrial mRNA is similarly poorly understood. In cases where multiple transcripts are generated it is not clear how many are translated. The 5' leader sequences of many transcripts contain numerous AUG codons, some out of frame, which presumably are not recognised as initiation sites, so there may be some mechanism by which the ribosomes select the correct site. A ribosome binding site analogous to the bacterial Shine-Dalgarno sequence has been proposed, and it has been noted that the initiation codon is usually followed by an adenine and that there are often many adenine residues between the RBS and the initiation site (Dawson *et al.* (1984), Schuster and Brennicke 1986). None of these features is ubiquitous, and their significance is unknown.

As the selection of the initiation codon is not understood, when constructing the transformation vectors used in this study, the CAT gene was fused to a site within the coding region of *coxI* (section 3.2). The entire untranslated leader sequence and the authentic translation initiation site were thus hopefully preserved, allowing CAT to be synthesised as a fusion protein.

It was not known whether CAT would be active if translated as a fusion protein, and in addition, such a translational fusion would have been difficult to construct with the *P. hybrida* ATP9-1 gene. Instead, in the construct employing the sequence upstream of *atp9* -1, the initiation codon of *cat* was positioned at almost the same distance from the ribosome binding site as the corresponding codon of the wild type ATP9 gene. Although the distance between these two points is similar in the wild type and chimaeric genes, the sequence between them differs. However, in a comparison of the ATP9 genes of tobacco and *Petunia* , the sequence in this region was found to vary, in contrast to the sequences immediately flanking it which are well conserved (Figure 3.14); thus the sequence alterations in this region of the chimaeric gene may also be tolerated.

The details of mitochondrial transformation vectors employing these sequences, and the method of their construction are described in the following sections.

3.2 Construction of the Transformation Vectors

3.2.1 Construction of Plasmids pUPS92J, pUPS92E and pSCOX920 Containing Translational Fusions Between *coxI* and *cat*.

It was decided to construct three basic vectors using the putative promoter region of *coxI* from maize. Plasmid pUPS92J was designed to express *cat* from the promoter of *coxI* from N-type mtDNA, and pSCOX92O was designed to express it from the promoter of *coxI* from S-type mtDNA. Plasmid pUPS92E contains the N-type promoter in inverse orientation relative to *cat* to act as a negative control. The cloned fragments of DNA that were used to construct these plasmids are described below.

A. Identification and Description of the Component DNA Fragments.

A Promoter Sequence from N Type Maize mtDNA.

The initiation sites for transcription and translation of maize *coxI* are situated on a 327 bp *Ava*II fragment from N-type mtDNA. This fragment was termed UPS. As shown in Figure 3.5 A and B, it is contained within an *Msp*I fragment which has been inserted into the *Acc*I site of M13 mp8 to produce the strain m3a3 (Isaac *et al.* 1985b). The internal *Ava*II fragment extends upstream from 12 bases into the *coxI* coding sequence to include both transcription initiation sites and 163 bp beyond the most distal of these sites (Figure 3.5 C). Fusion of this sequence to the *P. mirabilis* CAT gene will result in a short N-terminal extension to the enzyme, and should provide the gene with the sequences necessary for expression in plant mitochondria.

Figure 3.5

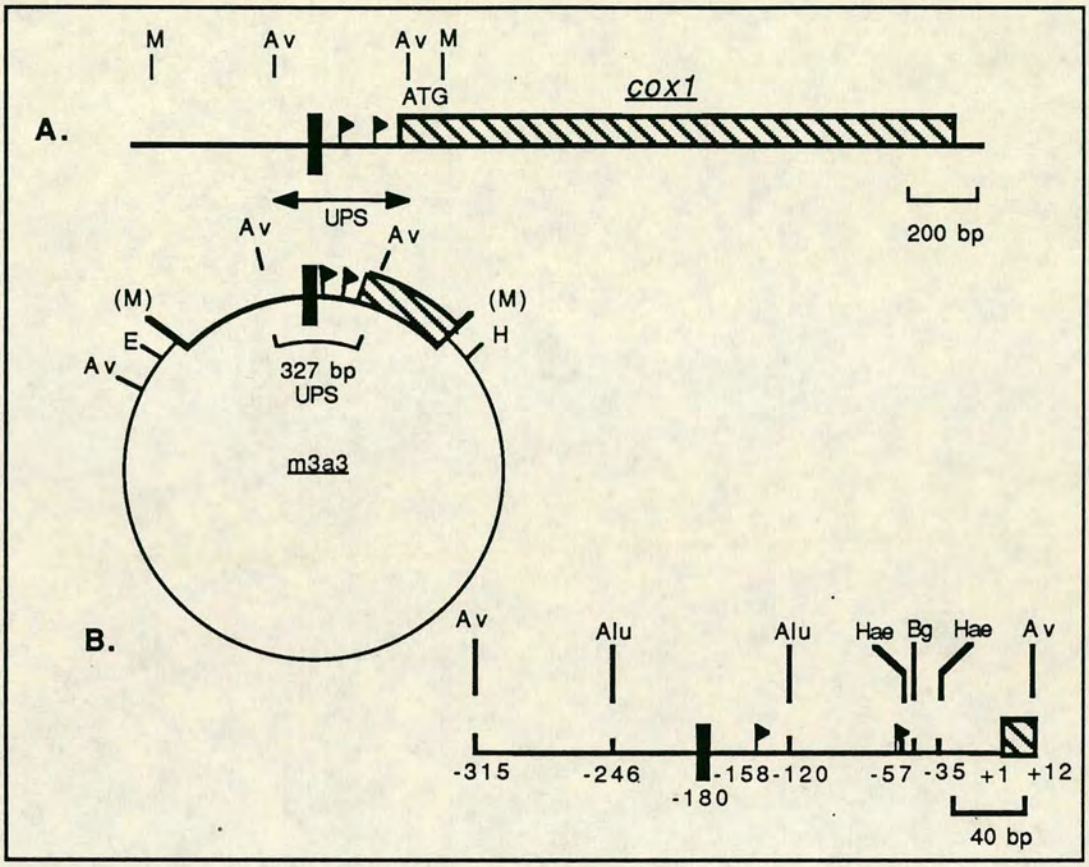
The Origin and Sequence of the UPS Promoter Fragment.

A. The genomic organisation of *coxI* in N-type maize mtDNA is shown. The 5' ends of the two transcripts are shown (flags) upstream from the coding region (hatched box), and the recombination site with homology to the S-TIR at which the N- and S-type sequences diverge is indicated (black box). The origin of the *Msp*I fragment cloned in m3a3 and the 327 bp *Ava*II fragment used in this work (UPS) are shown.

B. is a map (not to scale) of m3a3 showing the *Msp*I insert and *Ava*II sites. The UPS fragment is indicated and shown in greater detail to the right. Its sequence has been derived by Dr. P. Isaac (personal communication), and the coding strand is shown in **C.**

C. The predicted translation product from the portion of the *coxI* ORF present in UPS is shown in three letter notation beneath the nucleotide sequence of the coding strand. The potential ribosome binding site (-RBS-), transcript termini, the 20 bp sequence homologous to the S-TIR (dashed box) and the promoter consensus (boxed) are shown. M, Av, E, H, Alu, Hae and Bgl indicate recognition sites for *Msp*I, *Ava*II, *Eco*RI, *Hin*dIII, *Alu*I, *Hae*III and *Bgl*I respectively.

Figure 3.5



C.

-315

5' GACCCA ATGAGTAACT ACGCCATGTT ACGAATAAAA GCGAGGGAAA -271

ATCGGATGAG CAATCTTTTG CAGCTATATG AAATGCACTT TGGAAAATCA -221

Alu

GGTCTTGAGG ACGAAAAACA CGATAAAATG GACTTGCCTA CTTTTGCACC -171

GAAGAAACTC ATAAGTAATG CAATTTCCGA GGACACGAAC GGAAGTAAGC -121

Alu

TGTTTTTGGT ATTCGTATCG GAATTAGCCA GAAGAAGAAA CCATTTTCAC -71

TGCCCTTCAT TCTTTGGCCT GCCTGCCTTA GTGGCCCCTC TCTGATAAGG -21

Hae Bg Hae

TTTTCAAAAC GAAAAAAAAA ATG ACA AAT CTG -3'

RBS

Met Thr Asn Leu

+1 +12

The *Ava*II site 12 bases into the COXI encoding sequence was used also to generate the 3' end of the mtDNA fragment used to construct fusions between *cat* and sequences upstream of *coxI* in S-type mtDNA (S-*coxI*). Thus in the plasmids subsequently generated with the N- and S-type *coxI* fragments the sequences upstream of *cat* were identical up to the point of divergence between N and S mtDNA, 17 bp beyond the 5' terminus of the most distal transcript (Figure 3.9B)

Owing to the rearrangement of sequence relative to N-type mtDNA, the upstream *Ava*II site used to generate the 5' end of UPS is clearly not available for use in this construct. As explained in section 3.1.3, the region upstream of *coxI* in S-type mtDNA has been replaced by a series of different sequences all variously derived from the S plasmids. In one variant isolated by Isaac *et al.* (1985b) and shown in Figure 3.6 A, *coxI* was flanked by an undetermined length of a sequence that is found in N-type mtDNA adjacent to the 5.27 kb repeat. Although the nucleotide sequence of this region of the N genome was known when these plasmids were being constructed (personal communication from Dr. D. Lonsdale), it was uncertain how much of it was present upstream of *coxI* in S and, therefore, which restriction sites were available. I decided to map this region with restriction endonucleases using the known sequence around the 5.27 kb repeat as a guide.

Figure 3.7 B is a diagram of plasmid pHSB3 which is a derivative of the cloning vector pAT153 and contains a 4.8 kb insert encoding the whole of S-*coxI* including more than 1.5 kb upstream. This plasmid was used to map S-*coxI* and to identify a suitable restriction site within the sequenced region to act as the upstream border of mtDNA in fusions to the *cat* gene.

If the whole of the upstream sequence in pHSB3 corresponds to the sequence flanking the 5.27 kb repeat of N-type mtDNA, there should be *Cla*I sites at 470 bp and 830 bp upstream of the divergence point, and an *Eco*RI site 1486 bp upstream (Figures 3.6 A, and 3.7 B). As figure 3.7 A shows, the *Eco*RI site is not present, but *Cla*I cuts at positions consistent with both the expected sites. It was inferred that the sequence of pHSB3 diverges from that of the known 5.27 kb repeat region beyond the most distal *Cla*I site, so the *Cla*I site closest to *coxI* was selected for further cloning steps. Houchins *et al.* (1986) have subsequently confirmed the presence of this site by DNA sequencing upstream of S-*coxI*. The map and sequence of this *Cla*I to *Ava*II fragment, termed SCOX are shown in Figure 3.6 C and D.

Figure 3.6

The Origin and Sequence of the SCOX Promoter Fragment.

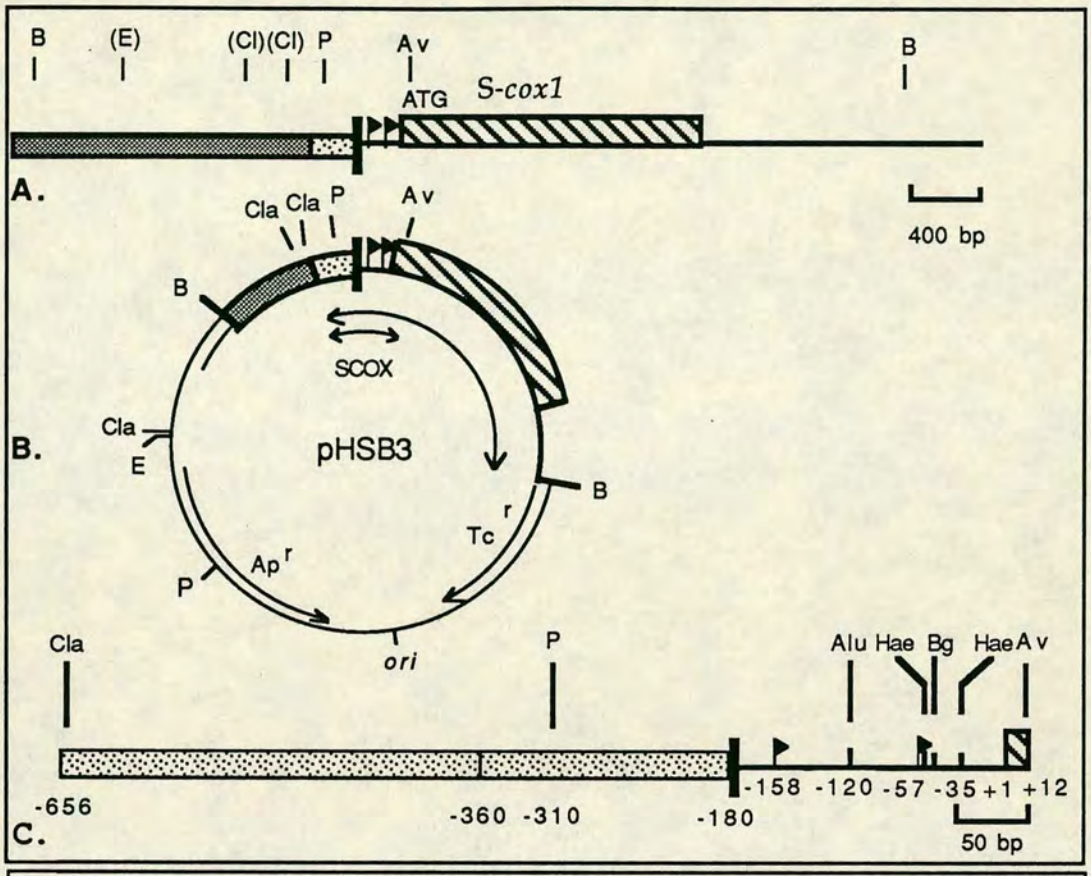
A shows the genomic organisation of one variant of S-*coxI* . The COXI encoding region, transcription and translation start sites and the sequence homologous to the terminal 20 bp of the STIR are indicated as in Figure 3.5. To the left of this the sequences introduced as a result of recombination at the STIR homology are shown; the stippled box represents the remainder of the 186 bp STIR sequence and other sequence normally found adjacent to the N-type 5.27 kb repeat; the shaded box represents the region whose sequence and origin had not been determined. The restriction sites indicated in brackets are predicted if the whole of this region is derived from the sequence flanking the 5.27 kb repeat. The only *Ava*II site that is shown is the one that was used to generate the 3' end of the SCOX fragment during vector construction.

B is a map of pHSB3 which consists of a 4.8 kb fragment of DNA from the region shown in A inserted at the *Bam*HI site of pAT153. The location of restriction sites is based on the mapping studies shown in Figure 3.7. The vector sequences are shown by a thin line, and its ampicillin and tetracycline resistance markers indicated by arrows with Ap^r and Tc^r respectively. *ori* represents the plasmid origin of vegetative replication. The 3.5 kb *Bam*HI to *Cla*I fragment initially isolated from the plasmid, and the 670 bp *Cla*I to *Ava*II fragment (SCOX) derived from it are shown by double headed arrows within the plasmid circle.

C is a detailed map of the 670 bp *Ava*II to *Cla*I SCOX fragment used for vector construction, and **D** shows its sequence. The entire region upstream of the recombination point has been shown to be derived from the 5.27 kb repeat on the basis of restriction mapping studies (Figure 3.7) and sequencing (Houchins *et al.* 1986).

All symbols are the same as in Figure 3.5, except P which represents a recognition site for *Pst* I.

Figure 3.6


B
(E)
(Cl)(Cl)
P
Av
ATG
S-cox1
B
A.
400 bp
Cla Cla
P
Av
B
SCOX
Cla
E
B.
pHSB3
B
Tc r
Ap r
P
ori
Cla
P
Alu Hae Bg Hae Av
-158 -120 -57 -35 +1 +12
-656
-360 -310
-180
C.
50 bp

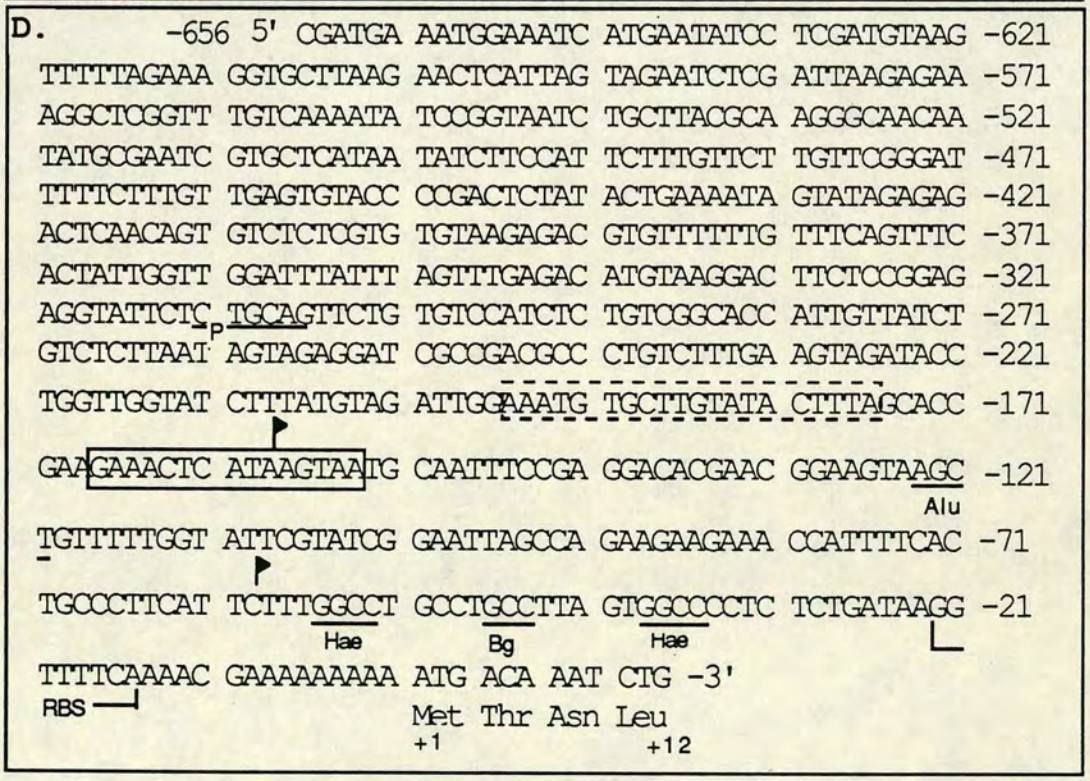

D.
-656 5' CGATGA AATGGAAATC ATGAATATCC TCGATGTAAG -621
TTTTTAGAAA GGTGCTTAAG AACTCATTAG TAGAATCTCG ATTAAGAGAA -571
AGGCTCGGTT TGTCAAAATA TCCGGTAATC TGCTTACGCA AGGGCAACAA -521
TATGCGAATC GTGCTCATAA TATCTTCCAT TCTTTGTTCT TGTTCGGGAT -471
TTTTCTTTGT TGAGTGTACC CCGACTCTAT ACTGAAAATA GTATAGAGAG -421
ACTCAACAGT GTCTCTCGTG TGTAAGAGAC GTGTTTTTTG TTTCAGTTTC -371
ACTATTGGTT GGATTTATTT AGTTTGAGAC ATGTAAGGAC TTCTCCGGAG -321
AGGTATTCTC TGCAGTTCTG TGTCCATCTC TGTCGGCACC ATTGTTATCT -271
P
GTCTCTTAAT AGTAGAGGAT CGCCGACGCC CTGTCTTTGA AGTAGATACC -221
TGGTTGGTAT CTTTATGTAG ATTGGAAAATG TGCTTGTATA CTTTAGCACC -171
GAAGAAACTC ATAAGTAATG CAATTTCCGA GGACACGAAC GGAAGTAAGC -121
Alu
TGTTTTTGGT ATTCGTATCG GAATTAGCCA GAAGAAGAAA CCATTTTCAC -71
TGCCCTTCAT TCTTTGGCCT GCCTGCCTTA GTGGCCCCTC TCTGATAAGG -21
Hae
Bg
Hae
TTTTCAAAAC GAAAAAAAAA ATG ACA AAT CTG -3'
RBS
Met Thr Asn Leu
+1
+12

Figure 3.7

Mapping pHSB3 to Determine the Presence of Potential Restriction Sites Upstream of S-*coxI*.

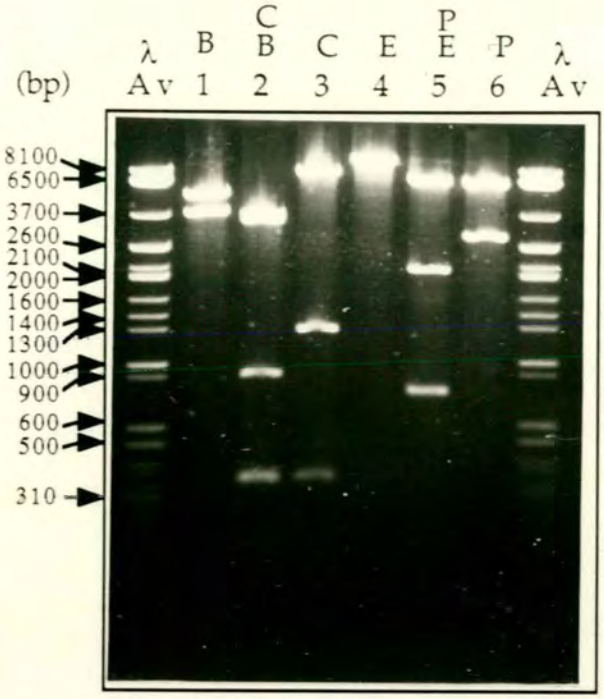
A. shows restriction digests of pHSB3 with various combinations of *Eco*RI (E), *Pst*I (P), *Bam*HI (B) and *Cla*I (C), separated by agarose gel electrophoresis, stained with ethidium bromide and photographed under U.V. illumunation. The origin of the significant fragments, that reveal the presence or absence of the potential *Eco*RI and *Cla*I recognition sites, are shown in **B.**

B. This is a diagram of pHSB3 drawn similarly to that in Figure 3.6 B. The sequence and restriction map of only the shaded segment of the plasmid was unknown. The 4.8 kb insert at the *Bam*HI site is released from the vector (3660 bp) with this enzyme as expected (lane 1). The only *Cla*I sites are close to the positions predicted from the 5.27 kb Repeat flanking sequence. Digestion with this enzyme releases a fragment of 350 bp and one of about 1.3 kb (lane 3) which can be further digested with *Bam*HI to give fragments of about 350 and 900 bp (lane 2). The *Eco*RI site of the 5.27 kb Repeat flanking region is not present. Digestion with this enzyme appears to linearise the plasmid at the recognition site within the vector, with no other fragment visible (lane 4). Also, the 2.7 kb *Pst*I fragment (lane 6) appears to be cut at only this point, yielding fragments of 750 bp and about 1.95 kb (lane 5), instead of 750 bp, 600 bp and 1.35 kb which would result from additional digestion at the potential site within the insert. The sequence upstream of the recombination point was assumed to diverge from that flanking the 5.27 kb Repeat beyond the distal *Cla*I site.

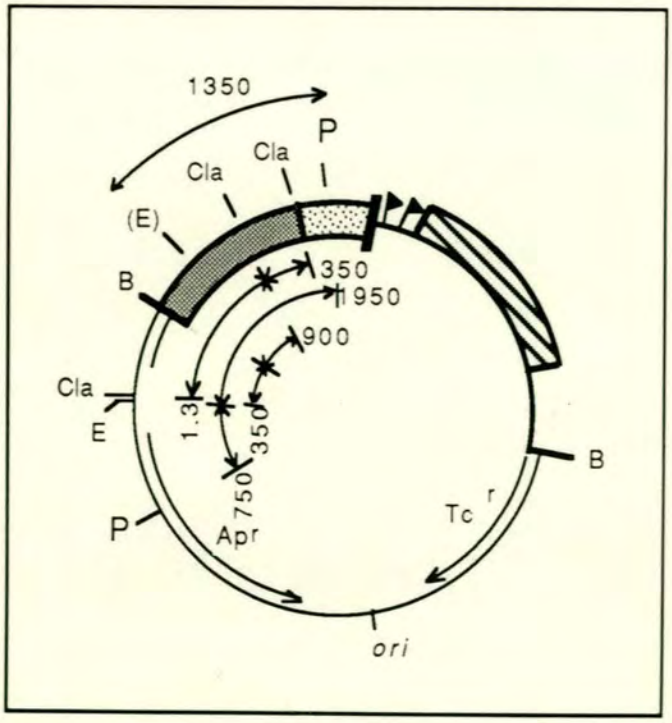
λ Av, indicates an *Ava*II digest of λ phage DNA.

Figure 3.7

A.


λ Av
B 1
C B 2
C 3
E 4
P E 5
P 6
λ Av
(bp)
8100
6500
3700
2600
2100
2000
1600
1400
1300
1000
900
600
500
310

B


1350
P
Cla
Cla
(E)
B
350
1950
900
Cla
E
1.3
350
750
B
P
Apr
Tc r
ori

A CAT Encoding Sequence from *Proteus mirabilis.*

The CAT gene from *Proteus mirabilis* PM13 was provided by Professor W. Shaw, University of Liecester. CAT is encoded in plasmid plco75 on a 5 kb fragment of genomic DNA cloned in the *Hin*dIII site of pUC13 (Charles *et al.* 1985b, Figure 3.8 A). The CAT gene is preceded by an *Alu*I site 5 bp upstream from the initial ATG codon, and is followed by a*Cla*I site about 90 bp downstream of the stop codon (Figure 3.8 B and C). Both of these sites are absent from the coding region of *cat* and were employed in the construction of fusions to *coxI* as described below.

Figure 3.8

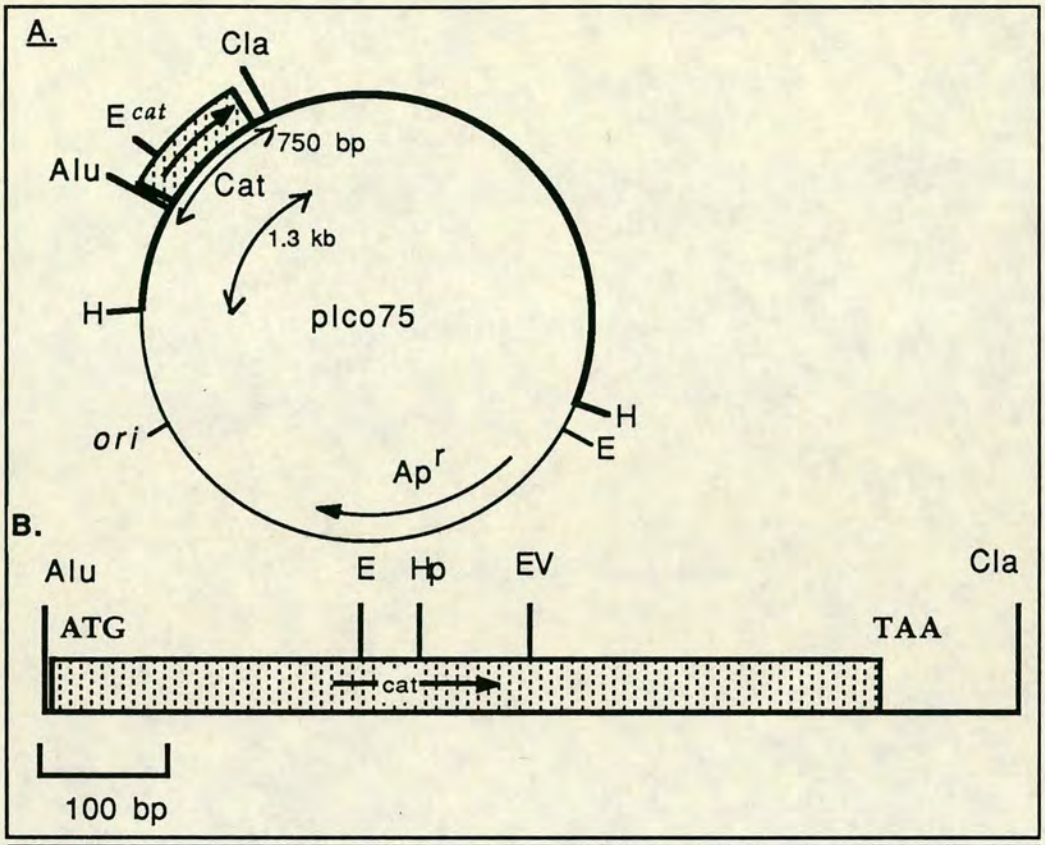
The Origin and Sequence of the Cat Fragment Encoding CAT in *P. mirabilis.*

A shows a map of pIco75 that encodes *cat* (Stippled box) of *Proteus mirabilis* . Vector sequences are shown by the thin line, and the chromosomal insert by heavy lines. the origin of vegetative replication (*ori*) and the ampicillin resistance marker (Ap^r)are shown. The 1.3 kb *Hin*dIII (H) to *Cla*I (Cla) fragment initially isolated, and the 750 bp *Alu*I (Alu) to *Cla*I fragment (Cat) containing *cat* that was derived from it are shown by double headed arrows.

B is an expanded map of Cat. The arrow indicates the direction of transcription. E, Hp and EV indicate recognition sites for the enzymes *Eco*RI, *Hpa*I, and *Eco*RV respectively. ATG and TAA represent the initiation and termination codons respectively.

C shows the sequence of the 750 bp Cat fragment, determined by Charles *et al.* (1985b) and in this work, (Chapter 4). The protein coding sequence is boxed, the initiation codon is indicated by Met and the restriction enzyme recognition sites by small boxes intercepted by either E (*Eco*RI), H (*Hpa*I) or V (*Eco*RV). The potential *Msp*I site that would be completed by the codon CGG is underlined.

Figure 3.8



C.

Met

```
CTAATATGGA CACAAAGCGC GTGGGATATA CTGTTGTTGA TCTATCCCAA +50
TGGGGACGAA AAGAACACTT TGAAGCATTT CAGTCTTTTG CTCAATGCAC +100
CTTTAGCCAG ACCGTTCAAC TGGATATTAC TTCATTATTA AAAACCGTAA +150
AGCAAAATGG GTACAAATTC TATCCGACAT TTATATACAT CATTAGCCTA +200
TTGGTAAATA AACATGCAGA ATTCCGCATG GCAATGAAAG ACGGGGAATT +250
GGTGATATGG GATAGTGTTA AGCCTGGGTA CAATATCTTT CATGAACAGA +300
CCGAAACATT TTCATCTTTA TGGAGTTACT ACCACAAAGA TATCAATCGT +350
TTTTTGAAAA CTTATTCAGA AGATATAGCA CAATACGGCG ACGATCTAGC +400
CTATTTTCCA AAAGAATTTA TCGAAAATAT GTTTTTTGTG TCAGCAAATC +450
CTTGGGTAAG TTTCACCAGT TTTAACTTGA ATATGGCGAA TATAAACAAT +500
TTCTTTGCCC CTGTATTTAC AATAGGTAAA TATTATACGC AAGGAGATAA +550
AGTTCTGATG CCACTAGCAA TTCAAGTGCA TCATGCCGTA TGTGATGGCT +600
TCCATGTAGG CAGATTACTC AATGAAATAC AGCAATACTG CGATGAGGGA +650
TGTAAATAAT CAATGGGACT TAAAGCACAT GTTTTTGTTC CACTCAAACC +700
TGTTCGCTAT CCTCTGTTTC GCACTTTTAT TGGATTATTT TAACAT 3' +746
```

B. Assembly of the Plasmids.

The strategy designed for assembly of pUPS92J, pUPS92E and pSCOX920 is outlined in Figure 3.9. The polylinker of pUC9 was chosen as a convenient source of restriction sites for creating an in-frame translational fusion. In this vector, the orientation of *cat* in the finished construct was such that it was not expressed from *lacP*. This promoter could potentially function in both *A. tumefaciens* and chloroplasts (Gruissem and Zurawski 1985), thus lowering the specificity of the selection procedure for mitochondrial transformants.

Initially, DNA of pIco75 was digested with *Hin*dIII and *Cla*I yielding fragments of about 1.3, 2.7 and 3.7 kb which were separated by electrophoresis through an agarose gel, and the 1.3 kb fragment containing *cat* was removed from the gel (Figure 3.8 A). This fragment was digested with *Alu*I to produce fragments of 0.57 and 0.74 kb. The larger of these was the desired *Alu*I to *Cla*I fragment (Cat) which was isolated from an agarose gel (Figures 3.8 B and 3.9, step1). This fragment was mixed with *Bam*HI digested pUC9 and the single stranded 5' overhangs filled in with T4 DNA polymerase before ligation (step 2).

Ligation products were used to transform JM83 cells, which were selected on ampicillin, and screened for β-galactosidase activity with X-gal. DNA was prepared from 38 β-galactosidase deficient clones, and insertion of *cat* screened for by digestion with *Eco*RI. One clone designated pCmP91 yielded fragments of about 0.5 and 3.0 kb as predicted if *cat* were present in pUC9 in the opposite orientation to that shown for pCmP92 in Figure 3.9. The same clone was digested with *Bam*HI, and produced fragments of about 2.7 and 0.75 kb (Figure 3.10A). This was expected because following the polymerase reaction, the 0.74 kb *Alu*I to *Cla*I Cat fragment should have had cytidine nucleotides at both 5' termini which regenerate the *Bam*HI sites of pUC9 upon insertion (Figure 3.11).

The CAT gene of *P. mirabilis* had been chosen in preference to that of *E.coli* because it has a CGC codon where the latter has CGG. This codon occurs adjacent to the *Eco*RI site in *cat*, forming the last three bases in the sequence 5' GAATTCCGC 3'. If the codon were CGG, it would form the sequence 5' CCGG 3' which is cleaved by *Msp*I. The *Msp*I restriction pattern of pCmP91 was determined to confirm that this site and the CGG codon were absent. The *Eco*RI site was found to cut as predicted (Figure 3.10A), which confirmed the presence of the first cytidine residue of the potential *Msp*I site. As shown in Figure 3.10B and D the vector is cut many times by *Msp*I, but the sites nearest the insert are 8 and 110 bp from its ends, within the *Sma*I site and beyond the *Hin*dIII site respectively (Yanisch-Perron *et al.* 1985). As expected *cat* was removed intact to give a fragment of about 900 bp that was not present in pUC19, and was cut by *Hin*dIII to give a fragment of 750 bp, and by*Eco*RV to give fragments of between 400 and 500 bp, confirming the absence of both the *Msp*I site and the CGG codon.

Figure 3.9

Scheme for Construction of the Mitochondrial Transformation Vectors pUPS92J and pSCOX92O.

A. The 750 bp fragment (Cat) containing *cat* was isolated from an agarose gel [Step 1], and ligated into the *Bam*HI site (B) in the β-galactosidase gene (*LacZ*) of plasmid pUC9. All the ends of these molecules were made double stranded with T4 DNA polymerase (T4pol) prior to ligation [Step 2]. A plasmid (pCmP92) containing the insert in the desired orientation was eventually recovered by reversing the insert of pCmP91 (not shown) which contained Cat in the inverse orientation.

The mitochondrial DNA fragments UPS (Figure 3.5) and SCOX (Figure 3.6) were isolated from agarose gels, [Steps 3 and 5], and ligated into the *Sma*I site (Sm) of pCmP92 [Steps 4 and 6]. The terminal phosphates were removed from the pCmP92 molecules with calf intestinal phosphatase (CIP), and the termini of all the molecules were made blunt ended with T4 DNA polymerase. Insertion of these fragments generated plasmids pUPS92J and pSCOX92O which contain the desired translational fusion between *coxI* and *cat* .

B. shows the region of plasmids pUPS92J and pSCOX920 containing the new chimaeric genes, with the sequence encoding the fusion protein derived from COXI and CAT shown below. The segments of these chimaeric genes are shaded in similar fashion to all the previous diagrams. The two plasmids are identical except for the sequence between the recombination point and the 5' end of their mitochondrial DNA insert. The first 5 amino acid residues are encoded by sequence derived from *coxI* , and are identical to those at the N-terminus of COXI. The next four codons are derived from the remainder of the *Sma*I and *Bam*HI sites of pUC9 and from the 5 bp preceding the CAT coding region in the 750 bp CAT fragment. The next codon is the authentic initiation codon of the CAT gene. The regenerated *Bam*HI site (Figure 3.11) between the *coxI* and *cat* sequences is underlined.

ori ; origin of replication. Ap^r ; ampicillin resistance marker. Amino acids and nucleotides are shown in standard triple and single letter notation respectively. Restriction enzyme recognition sites are represented as in previous figures, with Sl, He, Al and Pv additionally indicating *Sal*I, *Hae*III, *Alu*I and *Pvu*II respectively. Brackets indicate that the site was destroyed upon ligation.

Figure 3.9

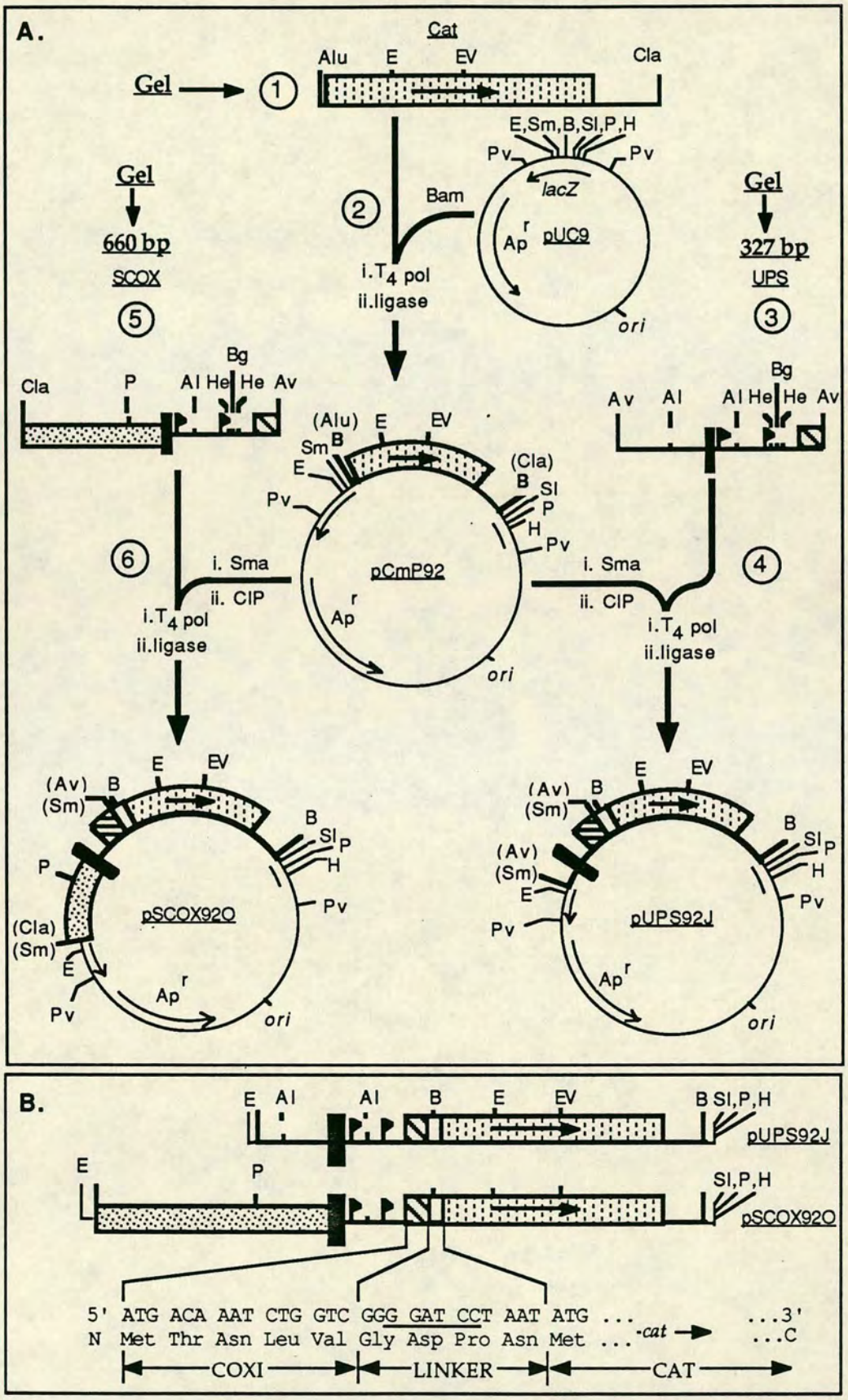

A.
Cat
Alu
E
EV
Cla
Gel
1
E,Sm,B,Sl,P,H
Pv
Pv
lacZ
Bam
2
Gel
660 bp
SCOX
5
i.T4 pol
ii.ligase
Ap r pUC9
ori
Gel
327 bp
UPS
3
Bg
Cla
P
Al He
He Av
Av
Al
Al He
He Av
Bg
(Alu)
E
EV
Sm B
E
(Cla)
B
Sl
P
H
Pv
Pv
6
i. Sma
ii. CIP
i.T4 pol
ii.ligase
pCmP92
Ap r
ori
i. Sma
ii. CIP
4
i.T4 pol
ii.ligase
E
EV
(Av)
(Sm)
B
B
Sl
P
H
P
Pv
(Cla)
(Sm)
E
Pv
pSCOX92O
Ap r
ori
E
EV
(Av)
(Sm)
B
B
Sl
P
H
(Av)
(Sm)
E
Pv
Pv
pUPS92J
Ap r
ori
B.
E
Al
Al
B
E
EV
B Sl,P,H
pUPS92J
E
P
Sl,P,H
pSCOX92O
5' ATG ACA AAT CTG GTC GGG GAT CCT AAT ATG ... ...3'
N Met Thr Asn Leu Val Gly Asp Pro Asn Met ... -cat → ...C
COXI
LINKER
CAT

Figure 3. 10

Restriction Endonuclease Digestion Analysis of pCm91 and pCm92.

Parts **A, B** and **C** show restriction endonuclease digestion products separated by agarose gel electrophoresis, stained with ethidium bromide and photographed under U.V. illumination. λ Av, indicates an *Ava*II digest of λ phage DNA.

A. *Eco*RI (E) and *Bam*HI (B) digests of pCmP91 plasmid DNA. *Bam*HI digestion releases the Cat fragment (760 bp) from the vector (about 2,700 bp), and the orientation of the Cat insert is revealed by the fragment of about 540 bp generated by *Eco* RI digestion at the asymmetric site within the Cat fragment. The origin and approximate size of these fragments are shown in part **D.**

B. This shows that, as expected, the Cat sequence lacks the potential *Msp*I (Msp) recognition site adjacent to the *Eco*RI recognition site. *Msp*I digests the vector sequence at many sites, but the two closest to Cat are within the *Sma*I (S) recognition site situated 8 bp from one end of Cat (part D.), and 110 bp beyond the *Hin*dIII site at the other end. Digestion of pCmP91 at these sites is expected to produce a fragment of 890 bp; a fragment consistent with this is present in pCmP91 (lane 2), but is not present in pUC19 (lane 1). This fragment is digested by *Eco*RV to give two fragments of between 400 bp and 500 bp as expected (lane 3); the vector sequence contains no recognition sites for *Eco*RV confirming that the 890 bp *Msp*I fragment contains Cat sequence. Similarly, the 890 bp *Msp*I fragment appears to be shortened as expected to about 780 bp by digestion with *Hin*dIII (lane 4). Had Cat contained the potential *Msp*I site, the *Eco*RV site would have been situated on a 530 bp *Msp*I fragment which would not have been modified by *Hin*dIII digestion.

C. Restriction endonuclease digestion analysis of pCmP92. The *Eco*RI restriction endonuclease fragment of about 230 bp reveals that the Cat sequence is reversed relative to pCmP91 (lane 1 and part **E.**). The structure of the clone was further confirmed by double digestion with *Eco*RI and *Hin*dIII (lane 2), *Msp*I and *Hin*dIII (lane 3), *Msp*I and *Bam*HI (lane 5) and with *Bam*HI alone (lane 4). All the expected bands were observed, and their origin is indicated in part **E.** The *Bam*HI digest reveals that the ends of the molecules remained intact during the digestion and ligation steps involved in generating this plasmid from pCmP91.

D. and **E.** show the relevant regions of pCmP91 and pCmP92 respectively. The polylinker cloning site of the vector (E to H) is in heavy lines. The Cat insert is boxed, the coding region is stippled, and its orientation shown by the arrow. The origin and size (bp) of the DNA fragments discussed above are shown beneath the map. Restriction endonuclease recognition sites are as above; M, Sl and P additionally indicate *Msp*I, *Sal*I and *Pst*I recognition sites.

Figure 3. 10

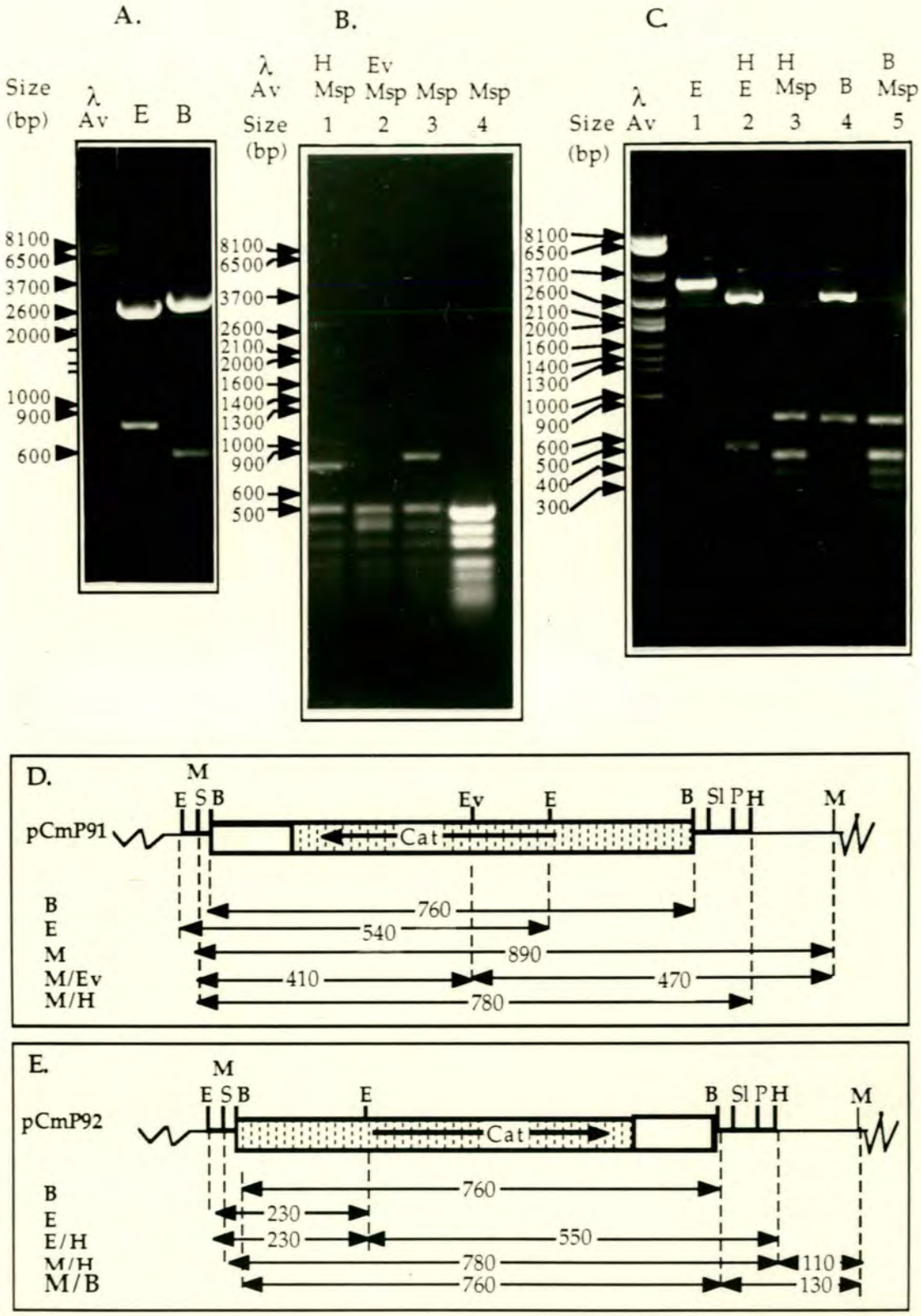
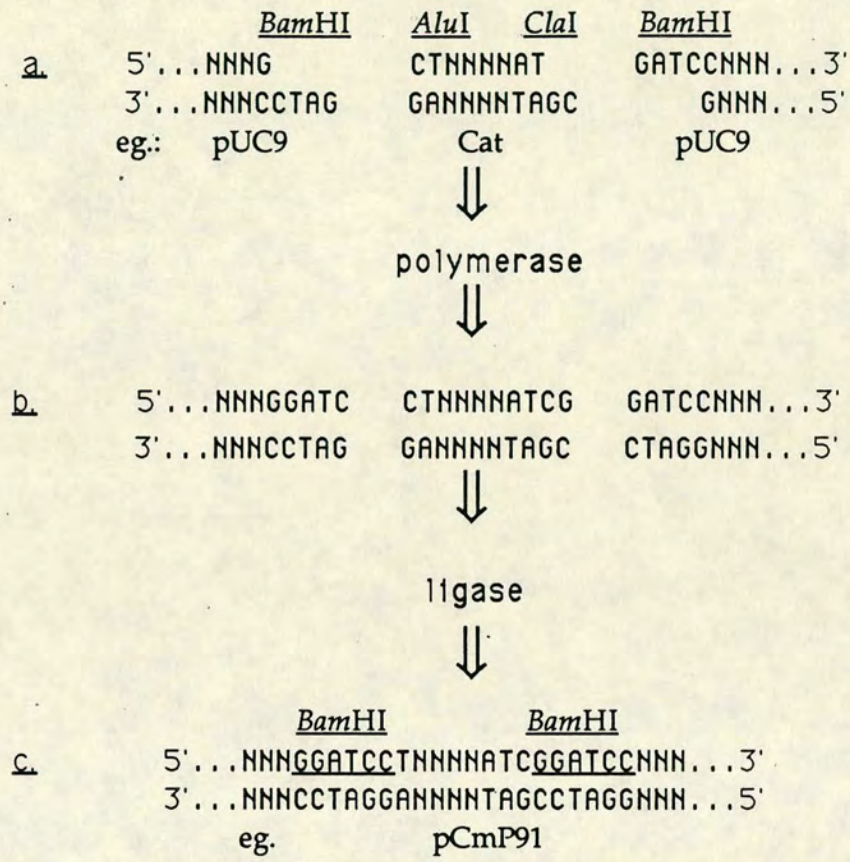

A.
B.
C.
Size (bp)
λ Av
E
B
H Msp
Ev Msp
Msp
Msp
1
2
3
4
H E
H Msp
B
B Msp
5
8100
6500
3700
2600
2100
2000
1600
1400
1300
1000
900
600
500
400
300
D.
pCmP91
M
E S B
Ev
E
B Sl P H
Cat
B
E
M
M/Ev
M/H
760
540
890
410
470
780
E.
pCmP92
230
550
780
110
760
130
E/H
M/B

Figure 3.11
Regeneration of *Bam*HI Sites Following Ligation.



a. represents a fragment of DNA which has been digested with *Alu*I and *Cla*I and a vector that has been digested with *Bam*HI. b. shows the effect of filling in the 5' overhangs to generate blunt ended molecules. c. shows the product of ligating these molecules to each other, and regeneration of the *Bam* HI sites. A, G, C and T are abbreviations for one each of the four deoxynucleotides, and N for any nucleotide.

Owing to the relative complexity of the subcloning procedure used to obtain pCmP91, I attempted to produce a plasmid with *cat* in the desired orientation by digesting pCmP91 with *Bam*HI and religating the digestion products. DNA prepared from 8 transformed colonies was digested with *Eco*RI, and a single clone produced fragments of about 0.2 and 3 kb as desired. The identity of the clone was confirmed by digestion with *Bam*HI, *Eco*RI with *Hin*dIII, and *Msp*I (Figure 3.10C and E). This clone was designated pCmP92, and its map is shown in Figure 3.9.

The next step was to insert the mtDNA fragments from m3a3 and pHSB3 into the *Sma* I site of pCmP92 (steps 4 and 6 in Figure 3.9).

Replicative form m3a3 DNA was digested with *Ava*II, and the smallest fragment, of about 0.33 kb, was isolated from an agarose gel (Figures 3.5 and 3.9, step 3). Also the 3.5 kb fragment generated by *Cla*I and *Bam*HI digestion of pHSB3 was similarly isolated (Figure 3.6B). This latter fragment is free from both the vector, which contains many *Ava*II sites, and the unknown sequence upstream of S-*coxI* , both of which would have made identification of the desired 0.66 kb *Cla*I to *Ava*II fragment more difficult. The 3.5 kb fragment was digested with *Ava*II, and the 0.66 kb SCOX fragment was isolated.

Plasmid pCmP92 was digested with *Sma*I, and treated with calf intestinal alkaline phosphatase. This vector was mixed with each of the fragments described above, T_4 polymerase reactions were performed to fill in the *Cla*I and *Ava*II termini, and the mixtures were then ligated (Figure 3.9, steps 4 and 6). JM83 cells were transformed with the ligation products, and clones were screened for insertion of mtDNA sequences by restriction enzyme digestion of isolated plasmid DNA.

Insertion of the 327 bp *Ava*II fragment of m3a3 into the *Sma*I site of pCmP92 increases the size of it's 230 bp *Eco*RI fragment to about 560 bp. A fragment consistent with this was seen in 6 of the 12 clones tested. The orientation of their inserts was determined by *Alu*I digestion, and their structure substantiated by double digestion with *Eco*RI and *Bam*HI (Figure 3.12). One of the clones with its *coxI* sequence oriented with similar polarity to *cat* was designated pUPS92J. One of the clones with *coxI* in the opposite orientation was designated pUPS92E.

Insertion of the 670 bp *Cla*I to *Ava*II fragment of pHSB3 into the *Sma*I site of pCmP92 introduces a second *Pst*I site. Digestion with *Pst*I was thus expected to yield fragments of about 1.1 and 3 kb, where pCmP92 yields a single one of 3.5 kb. One clone from 10 screened produced this 1.1 kb fragment, and it's identity was confirmed by digestion with *Eco*RI, *Eco*RI with *Pst*I, and *Eco*RI with *Bam*HI. This plasmid was designated pSCOX920, and it was shown by *Alu*I digestion that its *coxI* sequence was oriented with polarity similar to *cat* (Figure 3.13).

In parallel constructions, the N and S-type *coxI* fragments were inserted into the *Sma*I site of pCmP91. Two N and one S-type plasmids were obtained and named pUPS919, pUPS9110 and pSCOX911 respectively. Each plasmid had the *coxI*

Figure 3.12

Restriction Endonuclease Analysis of pUPS92J and pUPS92E.

Parts **A**, **B**, and **C** show plasmid DNA digested with restriction endonucleases, separated by agarose gel electrophoresis, stained with ethidium bromide and photographed under U.V. illumination. λ Av, indicates an *Ava*II digest of λ phage DNA.

A. This shows products of *Eco*RI (E) digestion of plasmid DNA isolated from 12 bacterial clones that potentially contain the UPS fragment inserted into pCmP92. This plasmid contains a 230 bp *Eco*RI fragment that would be increased to 560 bp by insertion of the 330 bp UPS fragment (part **D.**). A fragment consistent with this is seen in 6 of the 12 clones. Clones J and E (Asterisks) were shown to contain the UPS fragment oriented with similar and inverse polarity to Cat respectively (part **B.**) and were designated pUPS92J and pUPS 92E.

B. The orientation of the UPS insert was determined by digestion with *Alu*I (Al). This enzyme cuts within the *Hin*dIII (H) recognition sequence at the 3' end of the CAT gene (part **D.**), and at several other sites within the vector, but not within the Cat fragment. It cuts the UPS fragment 130 bp from one end and 70 bp from the other (Figure 3.5 B and C). Thus *Alu*I digestion of pCmP92 derivatives containing UPS will produce fragments containing the 770 bp Cat fragment plus either 130 bp (if the UPS sequence has the same polarity as Cat, shown in part **D.**) or 70 bp (if the UPS sequence has the opposite polarity). The plasmids in lanes 3 to 6, including pUPS92J (lane 6), have the larger fragment (about 900 bp), whereas pUPS92E (lane 2) generates the smaller 840 bp fragment. As a marker, pCmP92 was also digested with *Alu*I which cuts the vector about 95 bp 5' of Cat and thus generates a fragment of 865 bp intermediate between the two alternatives described above (lane 1). The digestion products were separated on a 1.8% gel.

C. The expected structure of the plasmids in lanes 3 to 6 of part **B.** was verified by double digestion with *Eco*RI and *Bam*HI. Digestion products included fragments consistent with the expected sizes of 340 bp, 220 bp, 550 bp (part **D.**) and the vector fragment of about 2,700 bp.

D. The relevant portion of pUPS92J including the UPS and Cat fragments is shown. It has been drawn similarly to Figure 3.9 B. The origin and size (bp) of the characteristic restriction endonuclease fragments discussed above are indicated below the diagram. Restriction endonuclease recognition sites are indicated as above, and B, Sl and P indicate sites for *Bam*HI, *Sal*I and *Pst*I respectively.

Figure 3.12

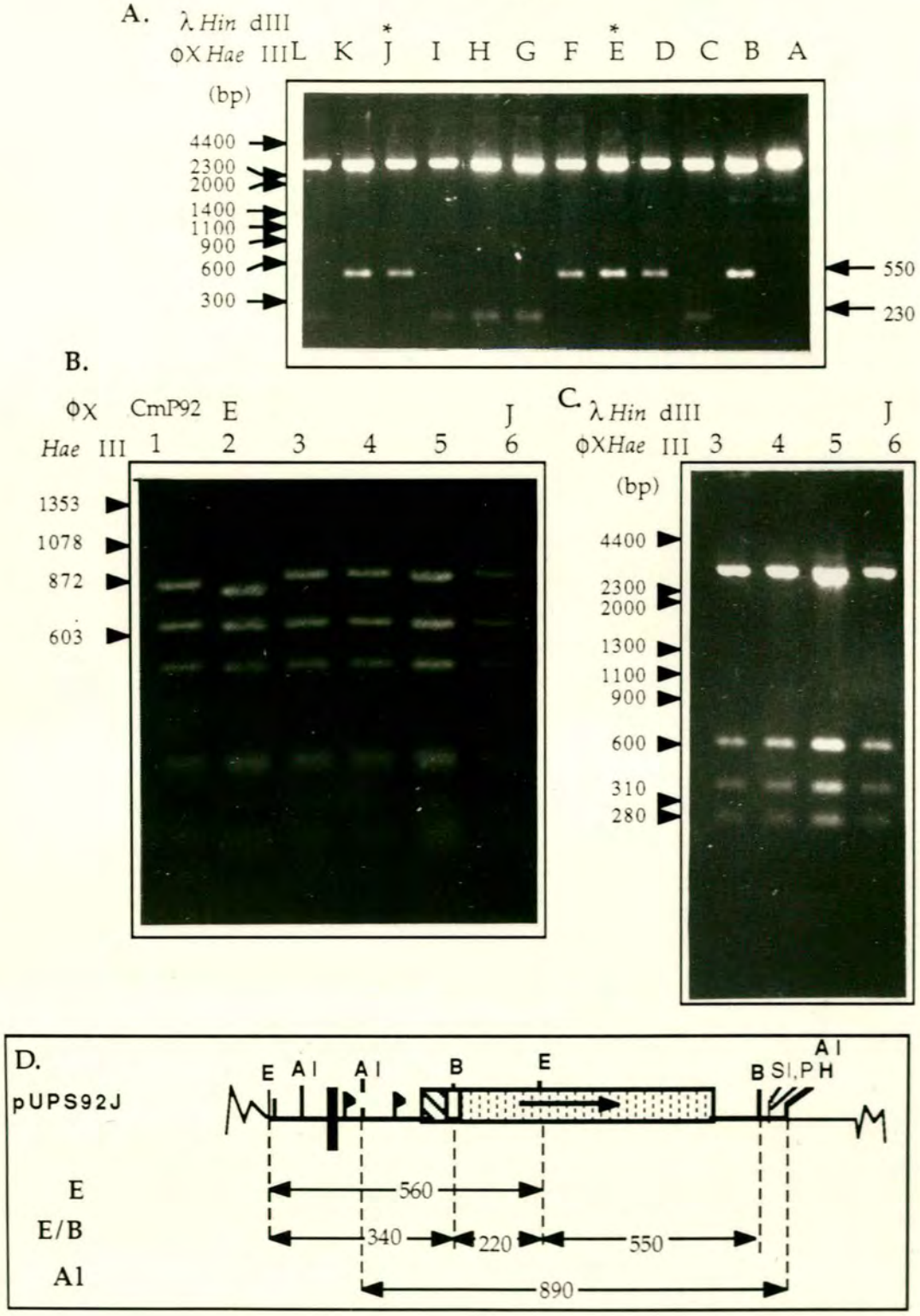

A.
λ Hin dIII
ϕX Hae III
L K J I H G F E D C B A
(bp)
4400
2300
2000
1400
1100
900
600
300
550
230
B.
ϕX CmP92 E J
Hae III 1 2 3 4 5 6
1353
1078
872
603
C.
λ Hin dIII J
ϕX Hae III 3 4 5 6
(bp)
4400
2300
2000
1300
1100
900
600
310
280
D.
pUPS92J
E AI AI B E B SI,P H AI
E
E/B
AI
560
340
220
550
890

Figure 3. 13

Restriction Endonuclease Digestion Analysis of pSCOX920 and pSCOX911.

Derivatives of pCmP91 and pCmP92 containing the SCOX sequence were called pSCOX911 and pSCOX92O respectively. The SCOX fragment was inserted in the same orientation in each plasmid, however, the Cat fragment is oriented inversely in each so only pSCOX92O contains the desired fusion (part **D.** and Figure 3.9B); pSCOX911 was retained as a potential negative control in plant transformation experiments. Parts **A, B,** and **C** are restriction endonuclease digestion products of plasmid DNA separated by agarose gel electrophoresis, stained with ethidium bromide and photographed under U.V. illumination.

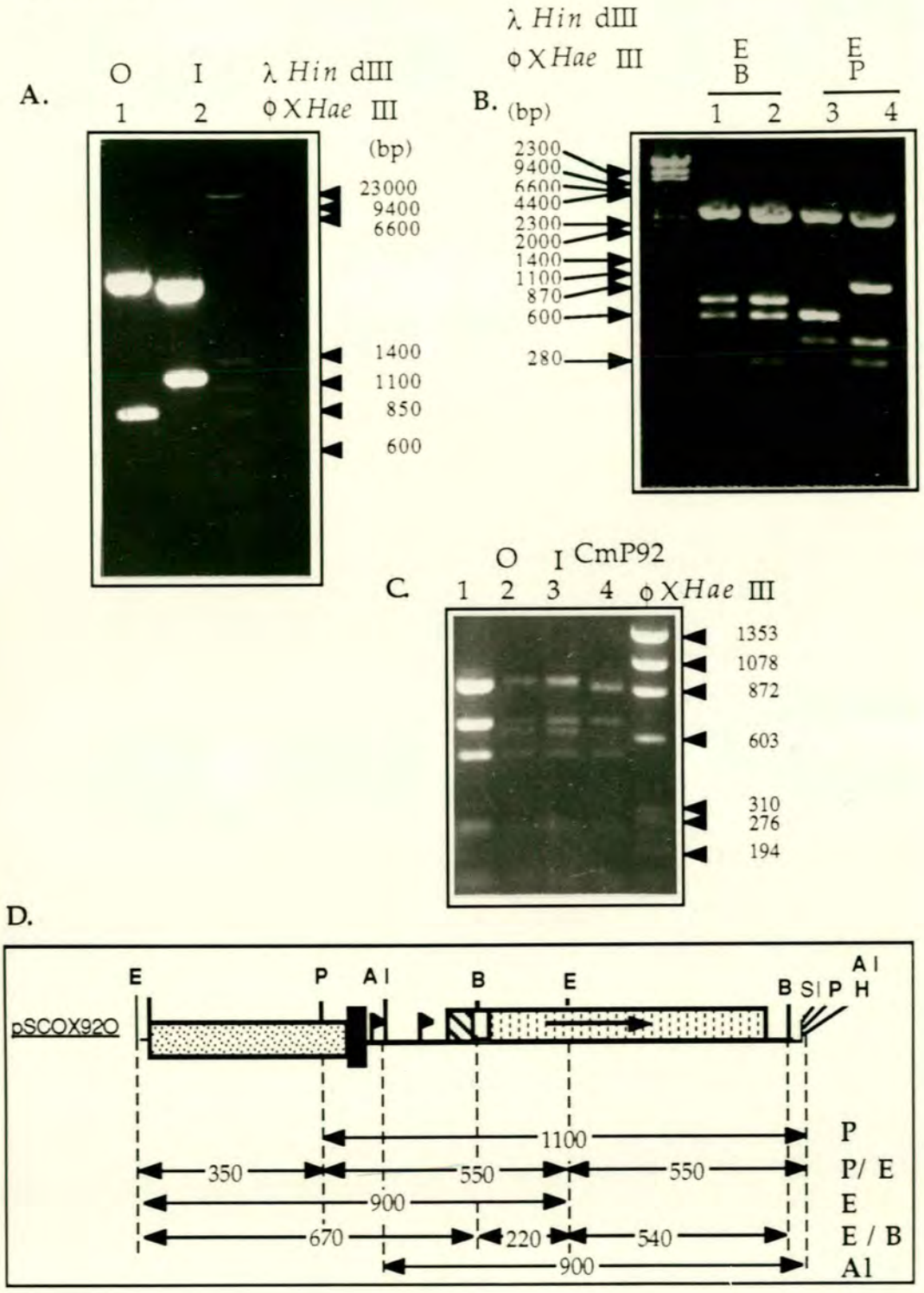
A. *Eco*RI (E) digestion products. In pSCOX92O, insertion of the 670 bp SCOX fragment into pCmP92 is expected to increase the size of the 230 bp *Eco*RI fragment to 900 bp (part **D.**, and lane 1, 'O'). In pSCOX911, insertion of SCOX into the 540 bp *Eco*RI fragment of pCmP91 (Figure 3.10 D.) is expected to increase its size to 1200 bp (lane 2, 'I').

B. The structure of pSCOX92O was confirmed by double digestion with *Eco*RI and *Bam*HI (B) (lane 1) and with *Eco*RI and *Pst*I (P) (lane 3). Fragments consistent with those expected were detected (670 bp, 220 bp, and 540 bp in lane 1, and 350 bp, 550 bp, and 550 bp in lane 3). The origin of these fragments is shown in part D. Lanes 2 and 4 are similar digests with pSCOX911 which are expected to produce fragments of 670 bp, 220 bp and 550 bp in lane 2, and fragments of 350 bp, 870 bp and 230 bp in lane 4.

C. *Alu*I (Al) digests of pSCOX920 and pSCOX911 (lanes 2 and 3 respectively). SCOX contains a single *Alu*I recognition site 130 bp from the 3' end and 530 bp from the other; thus fragments of either 900 bp or 1300 bp are expected depending upon the orientation of SCOX (770 bp+130, or 770 bp +530). Both plasmids generate fragments of about 900 bp (lanes 2 and 3) that comigrate with the equivalent fragment from a clone containing UPS oriented with the same polarity as Cat (lane 1), and that are larger than the equivalent fragment of pCmP92 (lane4). No fragment of 1300 bp was observed.

D. The relevant region of pSCOX920 containing the Cat and SCOX inserts is shown, and drawn similarly to Figure 3.9 B. The restriction enzyme recognition sites are indicated as above, and H and Sl additionally indicate sites for *Hin*dIII and *Sal*I. The origin and size (bp) of the DNA fragments discussed above are indicated beneath the diagram.

Figure 3.13


A.
O
I
λ Hin dIII
1
2
ϕX Hae III
(bp)
23000
9400
6600
1400
1100
850
600
B.
λ Hin dIII
ϕX Hae III
E
B
E
P
(bp)
1
2
3
4
2300
9400
6600
4400
2300
2000
1400
1100
870
600
280
C.
O
I
CmP92
1
2
3
4
ϕX Hae III
1353
1078
872
603
310
276
194
D.
pSCOX920
E
P
A I
B
E
B
S I
P
H
A I
1100
P
350
550
550
P/ E
900
E
670
220
540
E / B
900
A1

sequence in the same orientation as in pUPS92J, and was maintained in addition to pUPS92E as a potential negative control in transformation experiments.

The integrity of the fusion junctions in pUPS92J and pSCOX92O were confirmed later by DNA sequence analysis (see Chapter 4).

3.2.2 Construction of Plasmid pAPcat1 Containing *cat* Under Control of *atp9*-1 Promoter of *Petunia hybrida*.

Plasmid pATP 9 contains a 1.3 kb *Pst*I to *Bam*HI fragment containing the *atp9*-1 gene, 1 kb of upstream sequence and 50 bp downstream, inserted into the polylinker of pUC9. The termini of the three known transcripts of *atp9*-1 map within this plasmid (Young *et al.* 1986, Figure 3.14).

The coding region of *atp9*-1 was removed by digestion with *Eco*RI, which cuts 15 bp upstream of the initiation codon and also in the plasmid 3' of the mtDNA insertion. The 3.7 kb fragment containing the vector and putative promoter was isolated from an agarose gel (Step 1). In order that the initiation codon of *cat* was positioned 14 bp downstream from the *Eco*RI site, *cat* was isolated as a *Cla*I fragment from a derivative of pUPS92J constructed as follows. During the construction of plasmids described in section 4.5, it was necessary to convert the *Bam*HI sites of pUPS92J into *Cla*I sites. This was done by digesting the plasmid with *Bam*HI, filling in the 5' overhangs with T_4 polymerase and religating the modified fragments to give p92JBamFS (Figures 3.9 and 3.14 Step 2). The CAT gene of this construct was removed by digestion with *Cla*I which cuts the plasmid one nucleotide further away from the coding sequence than does *Bam*HI in pUPS92J. The 760 bp fragment was isolated from a gel, mixed with the 3.7 kb *Eco*RI fragment isolated above, and the 5' overhangs filled in with T_4 polymerase before ligation (Figure 3.14, Step 3). JM83 cells were transformed with the ligation products, and transformants screened for resistance to 4 µg/ml chloramphenicol on solidified YT medium. Plasmid DNA from resistant clones was digested with *Eco*RI. The *Cla*I to *Ec* RI junctions should regenerate the *Eco*RI sites upon ligation (Figure 3.15), and so there should be *Eco*RI sites flanking the insert, and one site within, producing fragments of 3.7, 0.55 and 0.23 kb upon digestion. One clone, designated pAPcat1, which had this structure was characterised further by digestion with *Hi* dIII and *Hpa*I and was used in later work (Figure 3.16)

In pAPcat1, *cat* may be expressed from the *lac* promoter, and this is probably responsible for the chloramphenicol resistant phenotype it induces in bacteria. As argued in section 3.2.2 this may lead to undesirable expression of the gene in *A. tumefaciens* and in chloroplasts. In this case, expression in *Agrobacterium* can be circumvented by omitting the *lac* promoter from the fragment transferred

Figure 3.14

Origin of the *P. hybrida* ATP9-1 Promoter Region, and Construction of pAPcat1

A shows the genomic organisation of the *Petunia hybrida* ATP9-1 gene. The coding region is shown as a black box, and the upstream region as a white box. The 5' termini of the three transcripts are shown by flags, and transcription proceeds from right to left to the termination site (Ter). The potential ribosome binding site (RBS) at the *Eco*RI recognition site (E) is shown. Av; *Ava*II. B; *Bam*HI. Xh; *Xho*I.

B shows the scheme used for construction of pAPcat1. Plasmid pUPS92J was initially digested with *Bam*HI the termini treated with T4 DNA polymerase (T4 pol), and the fragments religated (Step 2). Plasmid p92JBamFS was recovered and has an identical structure to its progenitor, except that its *Bam*HI sites (B), have been converted to *Cla*I sites (Cl). The 760 bp *Cla*I fragment containing the CAT gene was isolated from this plasmid and inserted between the *Eco*RI sites of pATP9-1 (Step 3). This plasmid contains a 1.3 kb *Pst*I to *Bam*HI fragment of mtDNA inserted at these sites (P and B, respectively) in the polylinker in *LacZ* (β-galactosidase) of pUC9 (Figure 3.9). EV; *Eco*RV. Hp; *Hpa*I. Pv; *Pvu*II. Sl; *Sal* I. H; *Hin*dIII. He; *Hae*III. *ori* ; origin of replication. Ap^r; ampicillin resistance marker.

C shows the sequences between the ribosome binding site (formed by the *Eco*RI recognition sequence, underlined) and the ATG initiation codon of the ATP9 genes from *P. hybrida* and *N. tabacum* and the CAT gene in pAPcat1.

Figure 3.14

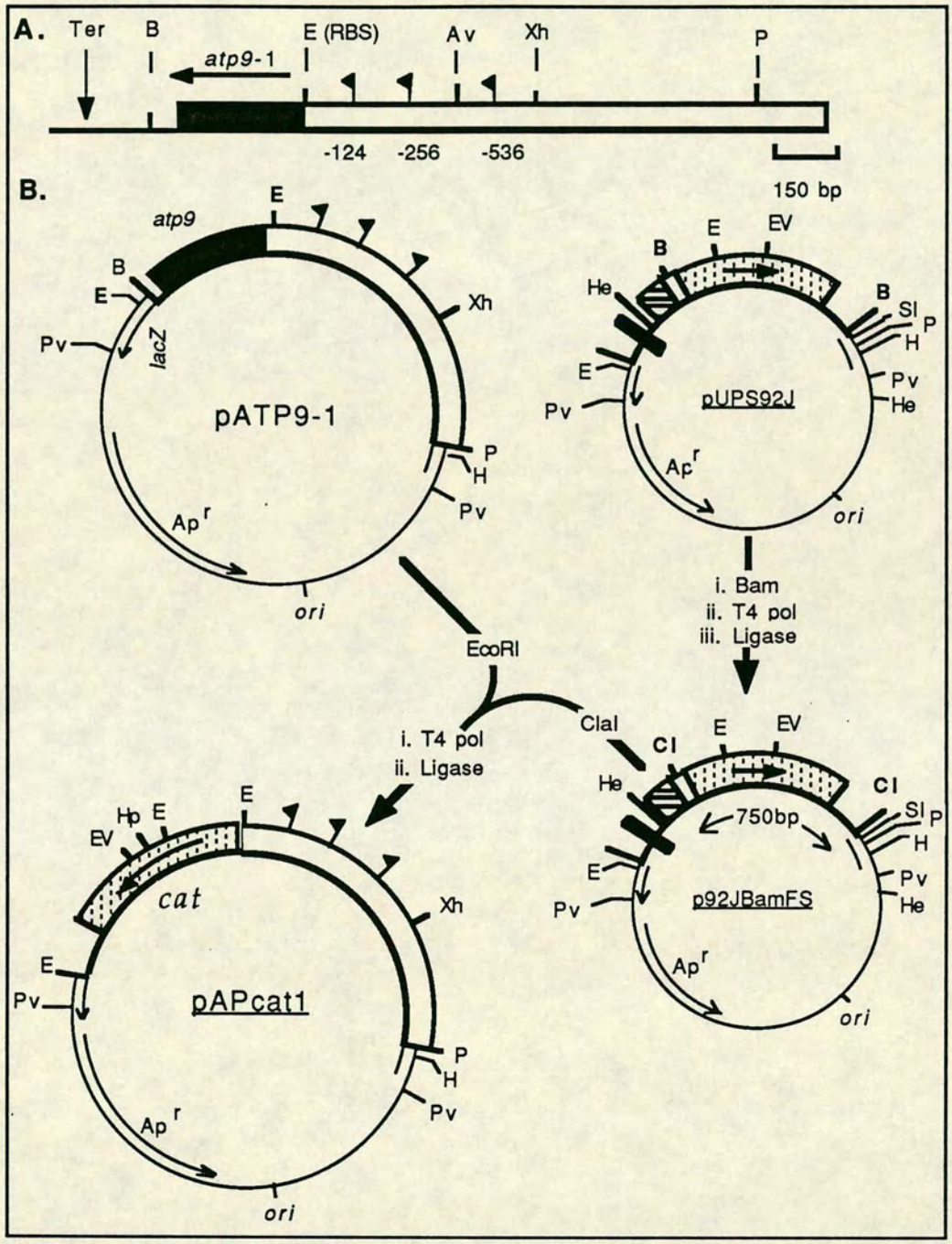
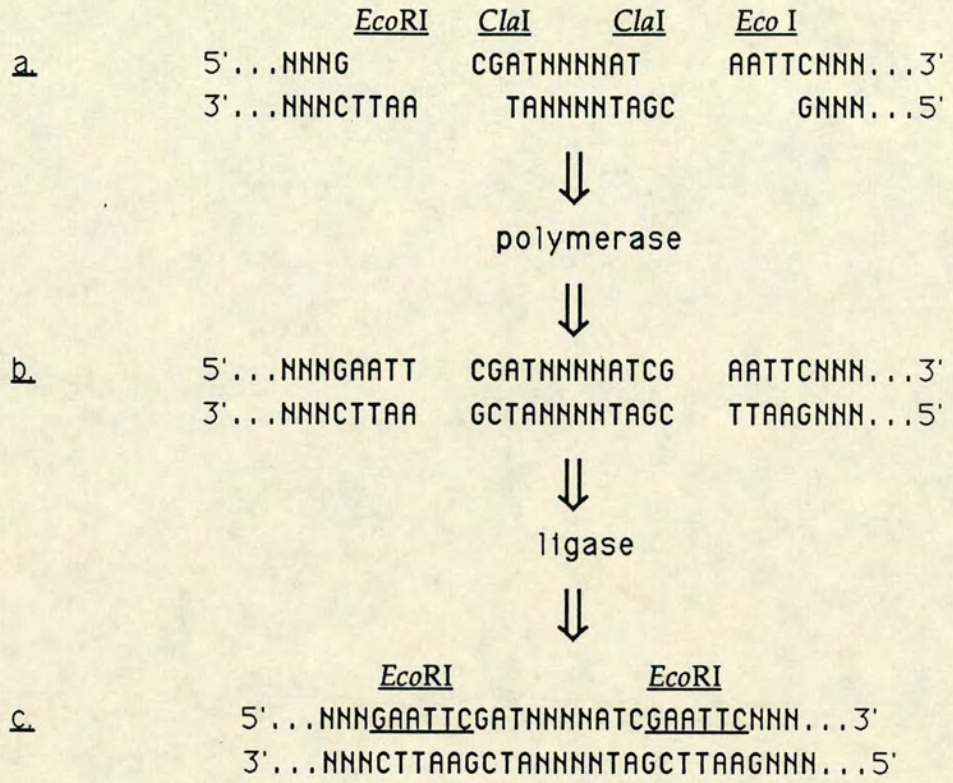

A.
Ter
B
atp9-1
E (RBS)
Av
Xh
P
-124
-256
-536
150 bp
B.
atp9
lacZ
pATP9-1
Ap r
ori
pUPS92J
EcoRI
i. Bam
ii. T4 pol
iii. Ligase
i. T4 pol
ii. Ligase
ClaI
cat
pAPcat1
p92JBamFS
750bp
C.
5'-GAATTCGAAAGTCGAT ATG——ATP9——► PETUNIA
5'-GAATTCGATCCTAAT ATG——CAT——► pAPcat1
5'-GAATTCTAAACTCGAG ATG——ATP9——► TOBACCO

Figure 3.15

Regeneration of *Eco*RI Sites upon Ligation to *Cla*I Sites.



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a. represents a fragment of DNA which has been digested with *Cla*I and a vector that has been digested with *Eco*RI. b. shows the effect of filling in the 5' overhangs to generate blunt ended molecules. c. shows the product of ligating these molecules to each other and the regenerated *Eco*RI sites. A, G, C and T stand for one each of the four deoxynucleotides, and N for any nucleotide.

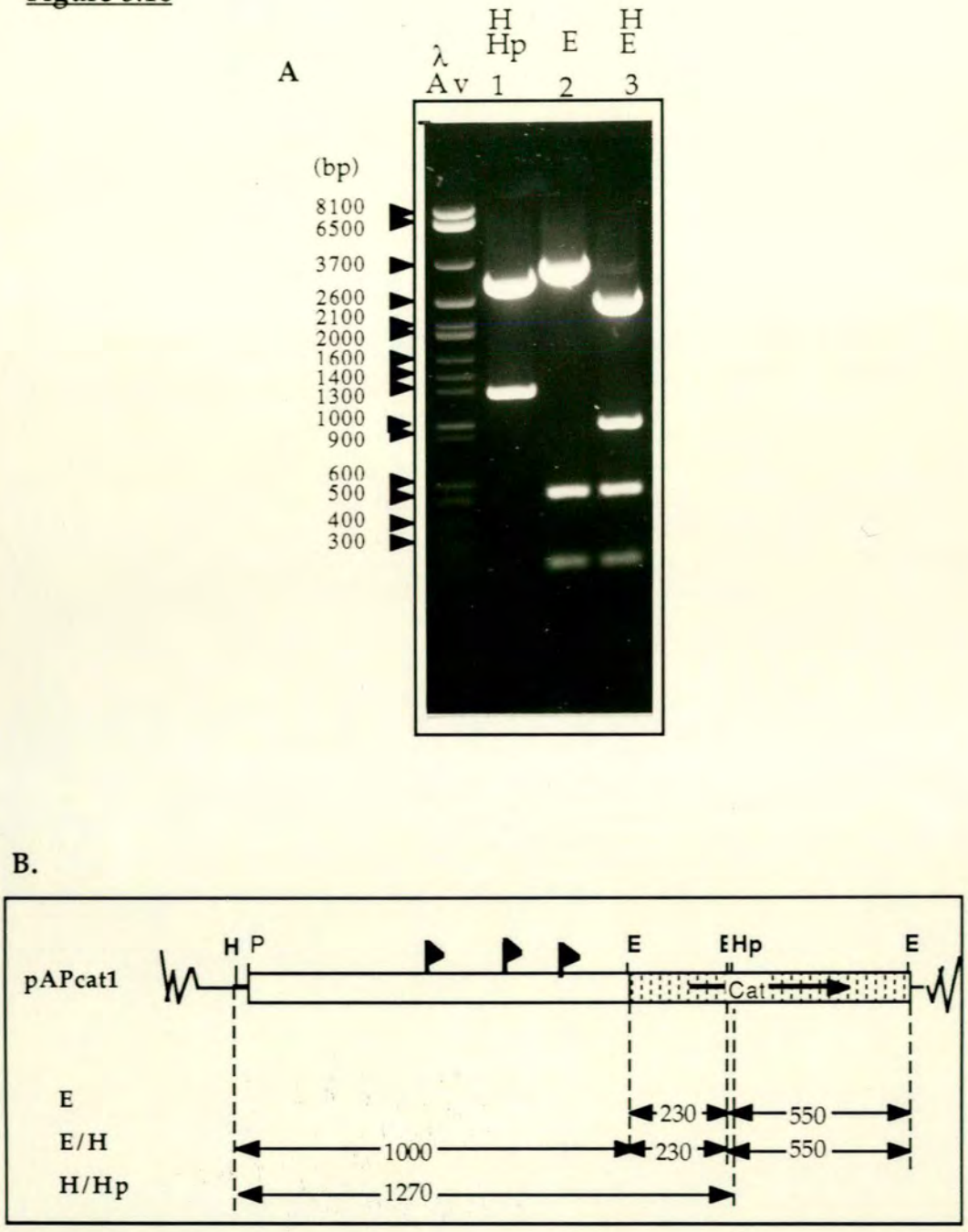
Figure 3.16

Restriction Endonuclease Digestion Analysis of pAPcat1.

A. The products of digestion of pAPcat1 plasmid DNA with *Eco*RI (E, lane 2), with *Eco*RI and *Hin*dIII (H, lane 3) and with *Hin*dIII and *Hpa*I (Hp, lane 1). Digestion products were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed under U.V. illumination. *Eco*RI is expected to produce fragments of 550 bp and 230 bp, *Eco*RI plus *Hin*dIII to produce fragments of 1000 bp, 230 bp and 550 bp, and *Hin*dIII and *Hpa*I to produce a fragments of 1270 bp as shown in part **B.** In addition, vector fragments of between 3 and 3.5 kb are expected.

B. Shows the relevant regions of pAPcat1 including the putative promoter sequence (open box) and the 770 bp CAT insert (stippled box). The flags indicate the sites at which the 5' termini of the three transcripts have been mapped Restriction enzyme recognition sites are indicated as above, and P additionally signifies a site for *Pst* I. The size and orientation of the fragments discussed above is shown below the diagram. If CAT had been inserted in the opposite orientation to that shown, the *Hpa*I and *Hin*dIII double digest would have resulted in a fragment of 1500 bp.

Figure 3.16


A
λ
Av
H
Hp
1
E
2
H
E
3
(bp)
8100
6500
3700
2600
2100
2000
1600
1400
1300
1000
900
600
500
400
300
B.
pAPcat1
H P
E
EHp
E
Cat
E
E/H
H/Hp
230
550
1000
230
550
1270

into Ti plasmid based transformation vectors, and at the time of construction of pAPcat1, it had become clear that transformation of chloroplasts was not sufficiently frequent to hinder recovery of mitochondrial transformants.

3.3 Conclusion.

In this chapter I have described the design and construction of plasmids that are intended to confer selectable chloramphenicol resistance to plant cells that harbour them in their mitochondria. Chloramphenicol was chosen as the most suitable of the selective agents available as the intracellular sites of action of the other antibiotics were not as well established. To confer resistance to chloramphenicol, a CAT gene from *P. mirabilis* was chosen because its codon usage was compatible with the proposed plant mitochondrial genetic code. The promoter region of the *Zea mays* COXI gene and the *Petunia hybrida* ATP9-1 gene were chosen to express the selectable marker. Plasmids pUPS92J and pSCOX920 contain gene expression signals from N and S-type maize mtDNA, and are designed to express *cat* as a translational fusion to *coxI*. Plasmid pAPcat1 is intended to express *cat* by transcription from the promoter region of a *Petunia hybrida atp9*-1 gene and by translation from the native initiation codon of *cat*. Plasmids in which the mitochondrial promoter sequences are missing or inverted relative to *cat* have also been constructed as negative controls.

In the following chapter the structures of the translational fusions are verified, and the functional properties of the plasmids in bacterial and plant cells are characterised.

CHAPTER 4.

STRUCTURAL AND FUNCTIONAL CHARACTERISATION OF THE MITOCHONDRIAL TRANSFORMATION VECTORS.

4.1 Introduction.

The effectiveness of the mitochondrial transformation vectors described in Chapter 3 relies on the exclusive expression of their CAT gene in mitochondria. It was necessary therefore to ensure that the fusions between *cat* and *coxI* were in frame, that the fusion protein retained CAT activity, and to determine whether *cat* is expressed when these transformation vectors are located in plant cell nuclei or in bacteria. Expression in the latter would indicate that problems may arise when plant cells are selected following transformation by *A. tumefaciens* or if transforming DNA enters the chloroplast (Gruissem and Zurawski 1985, Van Grinsven and Kool 1988).

4.2 Nucleotide Sequence Analysis of the Mitochondrial Transformation Vectors.

4.2.1 Nucleotide Sequence of the Fusion Site between *coxI* and *cat*.

Plasmids pUPS92J and pSCOX920 are intended to express translational fusions between *cat* and the five N-terminal amino acids of *coxI* , so it was necessary to check the integrity of their new fused reading frame. During construction of these plasmids, six T_4 polymerase reactions were performed to generate the fusion site, but the fidelity of only two of them could be verified by restriction endonuclease digestion (section 3.2.1 B). Reference to Figures 3.5, 4.1C and the map of pUPS92J in Figure 3.14 shows that the fusion region can be isolated on a 280 bp *Hae*III to *Eco*RI fragment. A 1 kb *Hae*III fragment was isolated, digested with *Eco*RI, and the desired 280 bp fragment was ligated into the *Eco* RI and *Hin*cII sites of M13 mp8. *Hae*III cuts *coxI* 47 bp upstream from the 3' *Ava*II site used in constructing pUPS92J, so the fusion site lies 67 bp away from the 3' end of the sequencing primer facilitating the determination of its sequence The sequence of this region of the clone was found to be as predicted (Figure 4.1).

When sequencing the *coxI* to *cat* fusion in pSCOX920, a larger, 850bp

fragment encompassing the whole open reading frame was used. One end of the fragment was generated by *Hae*III exactly as described above, and the other by *Alu* I which does not cut within the *cat* coding sequence, but within the *Hin*dIII site of the pUC9 polylinker beyond the 3' end of the gene (Figure 3.9B 4.1C). This fragment was cloned in the *Sma*I site of M13 mp19, and its orientation determined by restriction endonuclease mapping with *Eco*RI. A recombinant bearing the insert oriented with the junction site closest to the mp19 *Eco*RI and primer binding sites was isolated and its sequence determined by the Sanger method. Again the sequence was as predicted (Figure 4.1).

4.2.2 Correction of the Published *P. mirabilis* CAT gene Sequence.

During the analysis of pUPS92J described above, it was possible to determine the sequence of about 100 bp from the N-terminal coding region of *cat* . This agreed with that published by Charles *et al.* (1985b) except for 3 positions. Sequencing of the *cat* gene of pSCOX920 confirmed these differences. The polypeptide predicted from this alternative sequence differs by two amino acids from the published sequence of the *P. mirabilis* CAT gene. However at each of these positions the amino acid predicted from the new sequence is identical to the

Figure 4. 2
Comparison of the Predicted Translation Products from the Published and the newly Determined Sequences for the *P. mirabilis* CAT Gene, and their Similarity to the Types I and III Proteins.

Proteus	M D T K R V G **L L** V V D L	Published
	5'ATGGACACAAAGCGCGTGGG **TATATTG**GTTGTTGATCTA 3'	
	5'ATGGACACAAAGCGCGTGGGA**TATACT** GTTGTTGATCTA 3'	
	M D T K R V G **Y I** V V D L	This work
TypeI	M E K K I T G **Y I** T V D I	
TypeIII	M N **Y I** F K D V	

The amino acid sequences for the *P.mirabilis* and types I and III *cat* are indicated using the standard single letter notation. The published nucleotide sequence of the *P. mirabilis* gene, and that determined in this work are shown respectively below and above their deduced translation products. Nucleotides that differ between the two sequences are in bold type, and the affected amino acids with their codons are underlined.

Figure 4.1

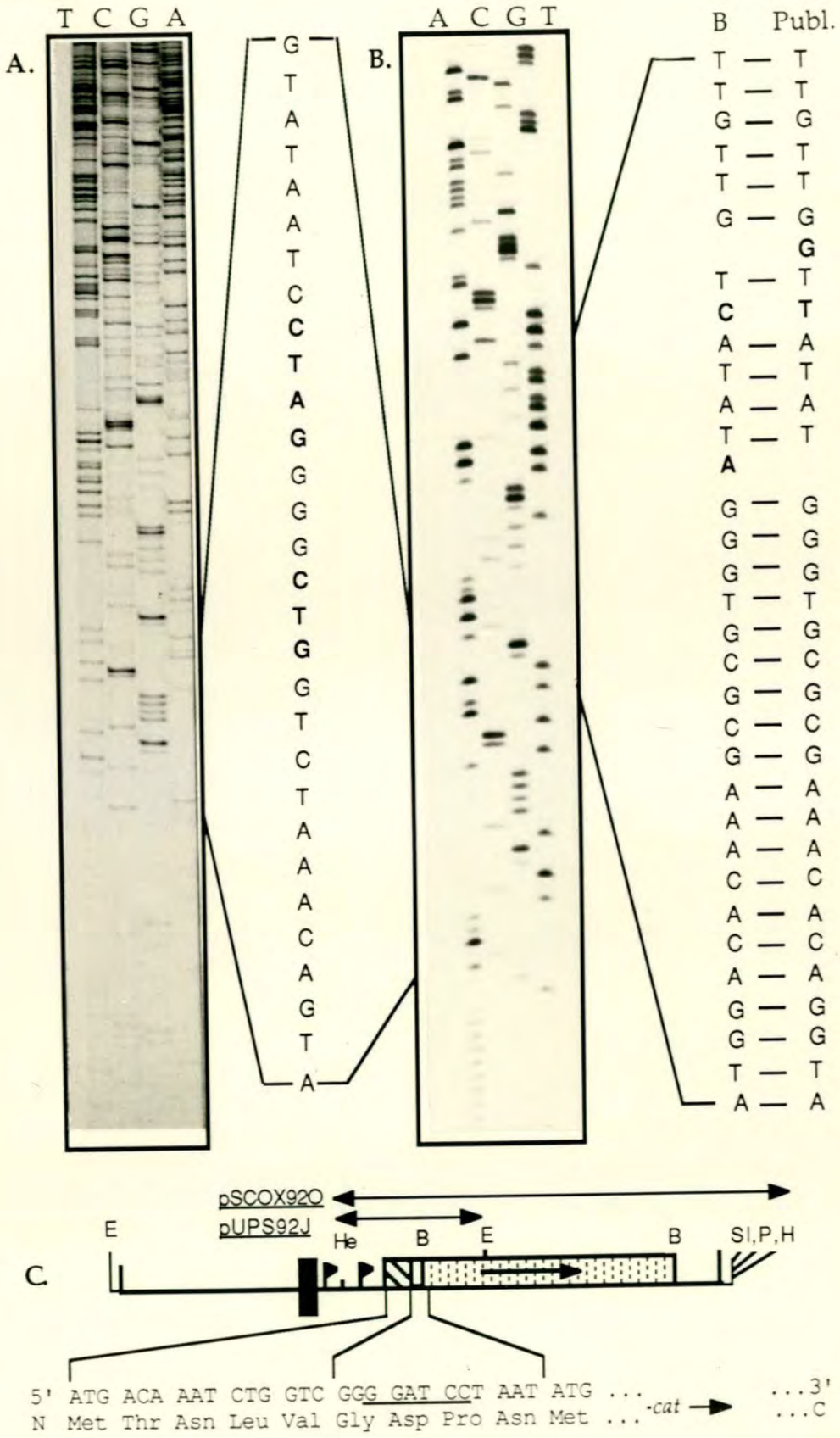
Nucleotide Sequence Analysis of the Junction Between Sequences Derived from *coxI* and *cat* in pUPS92J and pSCOX92O.

A. This shows the autoradiograph of the dried polyacrylamide gel used to derive the nucleotide sequence of the fusion between the COXI and CAT gene sequences in pSCOX920. The autoradiograph was generated by the Sanger dideoxynucleotide chain termination method as described in Materials and Methods. The letters T, C, G, and A at the top of the figure indicate, in standard single letter notation, the dideoxynucleotide that was incorporated into the reaction to generate the bands in each lane. To the right is shown the relevant sequence deduced from the autoradiograph, extending from the ATG initiation codon of *coxI* (bottom) to the initiation codon of *cat* (top). The nucleotides in bold face are those that resulted from the DNA polymerase reactions at the termini of the DNA fragments used for plasmid construction. The origin of the DNA fragment removed from pSCOX92O and inserted into M13mp19 is shown in part C.

B. This is similar to part **A**, but shows the autoradiograph used to derive the nucleotide sequence of the corresponding region of pUPS92J. In addition to the sequence of the gene fusion shown to the left, the region of the CAT gene sequence that was found to differ from that published by Charles *et al.* (1985b) is shown to the right. The CAT gene sequence derived from the autoradiograph is headed B and begins with the ATG codon at the bottom. The nucleotides that match the published sequence, headed Publ., are joined by a horizontal line. Nucleotides that do not match are in bold face. The origin of the DNA fragment removed from pUPS92J and inserted into M13mp8 is shown in part **C**.

C. This is a schematic representation of the chimaeric genes in plasmids pUPS92J and pSCOX92O, and is drawn similarly to Figure 3.9B. Double headed arrows show the origin of the restriction endonuclease digestion fragments that were used to generate the M13 mp8 and 19 derivatives from which the sequence shown in **A** and **B** was derived. In the sequencing reactions, chain elongation was in the direction from the *Hae*III recognition site to the *Eco*RI (pUPS92J) and *Hin*dIII (pSCOX92O) recognition sites. The expected nucleotide sequence and deduced polypeptide sequence is shown below the diagram.

Figure 4.1


T C G A
A.
G T A T A A T C C T A G G G G C T G G T C T A A A A C A G T A
B.
A C G T
B Publ.
T — T
T — T
G — G
T — T
T — T
G — G
G
T — T
C T
A — A
T — T
A — A
T — T
A
G — G
G — G
G — G
T — T
G — G
C — C
G — G
C — C
G — G
A — A
A — A
A — A
C — C
A — A
C — C
A — A
G — G
G — G
T — T
A — A
pSCOX92O
pUPS92J
E
He
B
E
B
Sl,P,H
C.
5' ATG ACA AAT CTG GTC GGG GAT CCT AAT ATG ... -cat → ...3'
N Met Thr Asn Leu Val Gly Asp Pro Asn Met C

one at the equivalent position in the types I and III sequences (Figures 4.1 and 4.2.). In fact, these amino acids are predicted only if all three DNA sequence changes are incorporated. For these reasons the sequence determined in this work was taken to be the correct one.

4.3 Chloramphenicol Resistance of *E. coli* Containing the Mitochondrial Transformation Vectors.

When the plasmids described above were constructed, it was not known whether the mitochondrial promoter sequences used would direct expression of *cat* in other systems. It has been shown that chloroplast genes can be expressed in *E. coli* (van Grinsven and Kool 1989), and it is well known, for example from the behaviour of shuttle vectors, that *E. coli* and *A. tumefaciens* will often express the same genes. Therefore, *E. coli* was used as a model system to estimate the level of expression that could be expected if these plasmids were in either *Agrobacterium* or in chloroplasts.

JM83 strains harbouring one of the plasmids under study were grown overnight in YT medium with ampicillin at 50 μg/ml, streaked out on YT plates containing increasing chloramphenicol concentrations or on 50 μg/ml ampicillin and incubated at 37°C overnight. Growth was estimated on each antibiotic regime and is shown in Table 4.1.

All plasmids examined expressed some CAT activity. Each plasmid carrying a mtDNA insert expressed more activity than the control, pCmP92, which contains *cat* but no mtDNA promoter. This implies that some portion of the mtDNA inserts are active, probably in transcription. The sequence TAAAATG which occurs about 200 bp upstream from the initiation codon of N-type *coxI* is homologous to the -10 consensus for *E. coli* promoters which is TATAATA/G (see Figure 3.5, and Siebenlist *et al.* 1980). This is separated by 13 and 39 bp from the sequences TTGAGG and TTGGAA which show homology to the -35 consensus sequence TTGACA. In S-*coxI* this region is replaced by sequence with no obvious promoter homology (see Figure 3.2). If transcription of *cat* in pUPS92J initiated here, the rearrangement in the S Type *coxI* sequence would provide an explanation for the lower resistance afforded by pSCOX920. As discussed below the resistance induced by even pUPS92J is low, possibly because the separation of the potential -10 and -35 sequences is far from the optimum of 17 bp; the sequences that do occur at this distance from each other are a poor match to the consensus (Siebenlist *et al.* 1980, Youderian *et al.* 1982).

. The level of expression achieved by these plasmids is relatively low when it is considered that the plasmids are in high copy number, that the single copy gene

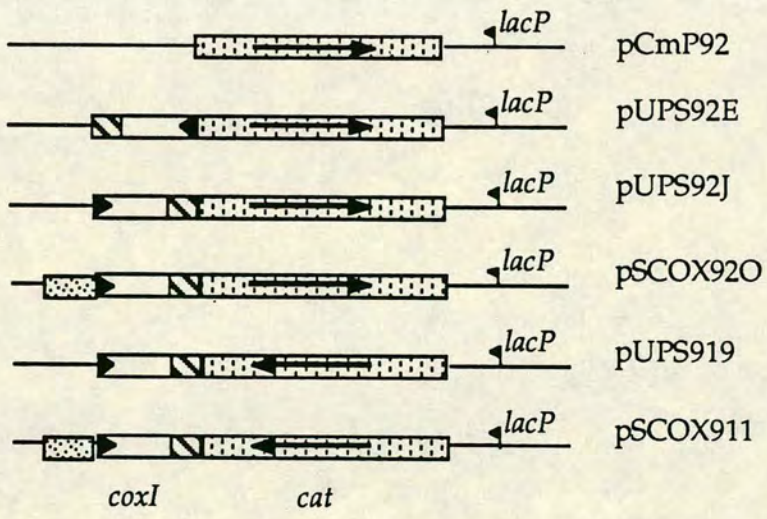
Table 4.1

Plasmid	Amp 50	Chloramphenicol 0	2	4	9	17	34	51	Comments
None		++	+-	-	-	-	-	-	
pCmP92	++	++	++	+-	-	-	-	-	No mtDNA
pUPS92E	++	++	++	++	+-	-	-	-	*coxI* reversed
pSCOX920	++	++	++	++	+-	-	-	-	S-Type promoter
pUPS92J	++	++	++	++	++	+-	-	-	N-Type promoter
pUPS919	++	++	++	++	++	++	++	++	*cat* reversed
pSCOX911	++	++	++	++	++	++	++	++	*cat* reversed

Each strain was grown on each antibiotic regime, and all those showing growth, either normal (++) or impaired (+-) are indicated. The numbers at the head of each column show the concentrations of ampicillin (Amp) and chloramphenicol in µg/ml. The plasmids have been described in sections 3.2 and contain the following inserts:

1. pCmP92 — *cat* , but no mtDNA, in polylinker of pUC9
2. pUPS92E — as pUPS92J, but with *coxI* promoter reversed
3. pUPS92J — as pCmP92, but with N type maize *coxI* promoter
4. pSCOX92O — as pCmP92, but with S type maize *coxI* promoter
5. pUPS919 — as pUPS92J, but with *cat* reversed
6. pSCOX911 — as pSCOX92O, but with *cat* reversed

These plasmids are represented below: the CAT gene is stippled and its orientation is indicated by the arrow; the COXI sequence is hatched, and the putative promoter indicated by the black triangle in the white box



on the *P. mirabilis* chromosome confers resistance to up to 500 µg/ml of chloramphenicol (Charles *et al.* 1985a), and that the highest levels of resistance observed in this experiment were conferred by pUPS919 and pSCOX911. In these plasmids, the orientation of *cat* is such that its transcription probably initiates from *lacP* which normally promotes transcription of *lacZ*. This promoter is not strongly inhibited by repressor in JM83. However, because the reading frame of *cat* is not in frame with *lacZ* in either plasmid, translation must begin from its own initiation codon. This lies internal to *lacZ* and has only weak Shine-Dalgarno homology upstream, so translation of the *cat* mRNA is probably impaired. None-the-less, these plasmids still confer the greatest resistance to chloramphenicol.

It is not possible from this data to draw any detailed conclusion about the relative efficiency of mitochondrial and bacterial promoter regions in *E. coli*. This is primarily because the *coxI* sequences responsible for transcription in *E. coli* are unknown, and because the sequences upstream of *cat* in pUPS92J and pSCOX920 are very different to those in pUPS919 and pSCOX911. Whilst such a comparison may be of general interest, it is not directly of importance here. Thus, it was concluded that *cat* will not be highly expressed from the mitochondrial transformation vectors in either bacterial cells or in chloroplasts. However, there will probably be some weak CAT activity which must be borne in mind, particularly when selecting cells transformed with *A. tumefaciens*.

Plasmid pAPcat1 was not included in this analysis because *lacP* is probably responsible for much of the CAT activity observed in bacteria harbouring this plasmid (section 3.2.2).

4.4 Expression of *cat* in Plant Cells Carrying the Mitochondrial Transformation Vectors in their Nuclei.

The strategy for obtaining mitochondrial transformants relies, initially at least, upon the use of established transformation procedures which are known to transfer at least some DNA to the nucleus. It must be accepted that mitochondrial transformation may be a much rarer event than nuclear transformation, so it was important to determine that the vectors used did not confer chloramphenicol resistance to plant cells when situated in the nucleus.

For this reason expression of *cat* from plasmids pUPS92J, pUPS92E and pSCOX920 was assayed in two systems in which plant nuclear genes are known to be active; firstly after deliberate stable nuclear transformation and secondly by analysis of transient expression.

4.4.1 Insertion of the Mitochondrial Transformation Vectors into the Tobacco Nuclear Genome.

The Mitochondrial Transformation Vectors Require a Selectable Marker for Nuclear Transformation.

As with most transformation systems, nuclear transformation of plant cells with purified DNA is sufficiently rare to require a selectable marker to recover the transformants. The mitochondrial transformation vectors that have been described so far could not have been relied upon to confer a selectable phenotype after nuclear transformation. An alternative, empirical, approach would have been to directly transform plant cells with the mitochondrial vectors, then to select transformants with chloramphenicol and determine the site of transformationin any resistant calli that emerge. However, this was not possible because a suitable selection scheme using chloramphenicol had not been established. There was therefore a necessity to provide the plasmids with a marker that would allow recovery of nuclear transformants, and it was decided that cotransformation with such a marker would achieve this most rapidly.

Cotransformation is said to have occurred when an organism is transformed by two distinct DNA molecules, and whilst rare with bacteria is common in mammalian cells (Scangos and Ruddle 1981). Czernilofsky *et al.* (1986a) had provided the initial evidence that it may be a common event in plant cells, and this has subsequently been confirmed by Wirtz *et al.* (1987) and Schöcher *et al.* (1986). They showed that when protoplasts are transformed with a mixture of two

different plasmids, a high proportion of cells selected for expression of one plasmid also contains the other, even if its presence has not been directly selected for. The cotransformed plasmids appear to be physically linked and integrated at the same site in genomic DNA (Czernilofsky *et al.* 1986a). Although it appears that plant cells are often and perhaps usually, transformed by more than one DNA molecule (for example see the references above, and also Depiker *et al.* 1985 and Deroles and Gardner 1988 for similar observations with *A. tumefaciens*), in what follows, the term cotransformation is used specifically to mean integration in one cell of sequence from two different plasmid species present during transformation.

Cotransformation of the Mitochondrial Transformation Vectors with the Nuclear Kanamycin Resistance Determinant of pLGV1103

Plasmid pLGV1103 expresses the NPTII gene (*nptII*) of Tn*5* in plant cells and has been used by Hain *et al.* (1985) and Czernilofsky *et al.* (1986a) to select kanamycin resistant transformants. If a mixture between this plasmid and one of the mitochondrial transformation vectors is used to transform plant cells, some of the kanamycin resistant nuclear transformants that result will contain the mitochondrial vectors in addition to pLGV1103. As a positive control for cotransformation, mixtures between pLGV1103 and pCAP212 were used; when integrated into tobacco cell nuclei, the latter plasmid has been shown to express *cat* from the 1' promoter of *A. tumefaciens* T-DNA (Velten and Schell 1985).

DNA of plasmid pLGV1103 was linearised at its unique *Hin*dIII site, and 20 µg of DNA mixed with an equal amount of each of pUPS92J, pUPS92E and pSCOX920 all of which had been digested with *Pvu*II (see Figure 3.9). As a positive control for cotransformation, 20 µg of plasmid pCAP212 was digested with *Pst*I and mixed with 20 µg of linearised pLGV1103. Plasmid pCAP212 will usually also confer kanamycin resistance to plant cells by expressing the NPTII gene of Tn*5* . This would clearly hinder its use in estimating the frequency of cotransformation of its CAT gene with the NPTII gene of pLGV1103. However, the NPTII gene unlike *cat* contains a *Pst*I site (Beck *et al.* 1982, Velten and Schell 1985) so it was inactivated by the *Pst*I digestion used during preparation of the sample.

Tobacco mesophyll protoplasts were transformed on three separate occasions by the PEG-calcium nitrate technique using these plasmid mixtures. On each occasion 20 µg of plasmids pCAP212 and pLGV1103 were digested as above and used individually in additional transformations as controls.

Transformed calli were selected with kanamycin, and all transformations in

which pLGV1103 (*nptII*) was present yielded between 100 and 1000 resistant calli although the exact number was not determined. In three experiments, only a single kanamycin resistant callus was recovered when pCAP212 (*npt-, cat*) was used alone, indicating that it's NPTII gene had been almost entirely inactivated. About 50 resistant calli from each cotransformation were transferred to solid media and, when large enough, several were assayed for CAT activity. Calli transformed with pLGV1103 (*nptII*) alone were assayed as negative controls, and those transformed in the presence of both pCAP212 (*npt-, cat*) and pLGV1103 (*nptII*) were assayed firstly to show that cotransformation was possible under the conditions used, and secondly to estimate its frequency. Examples of the assays are shown in Figure 4.3 and the results are compiled in Table 4.2.

Figure 4.3 shows that there is weak CAT activity associated with the negative controls. Although not shown here, this activity is also present in untransformed tobacco tissue, and represents a low level background activity. High levels of CAT activity were detected in about 60% of kanamycin resistant calli derived from protoplsts transformed with a mixture of pLGV1103 and pCAP212. These calli appear therefore to be cotransformants. The rest have activities within the range present in simple pLGV1103 transformants, so if they are cotransformants they are not detectably expressing *cat* . This figure of 60% is similar to that determined by Schöcher *et al.* (1986) who obtained a cotransformation frequency of 50% calculated also on the basis of expression of a non-selected marker, but using different plasmids and a higher ratio of non-selected to selected plasmid. Such consistency helps to justify the proposition that results obtained with pLGV1103 and pCAP212 reflect the frequency of cotransformation with pLGV1103 and the other plasmids. If this is the case, then from the data in Figure 4.3 it can be concluded that expression of *cat* from the mitochondrial transformation vectors in tobacco callus nuclei does not occur to an extent even approaching that achievable by pCAP212.

There are however two qualifying points to be made. Firstly, as there is a background CAT activity in pLGV1103 transformants and untransformed cells, the possibility of weak expression from the mitochondrial sequences is difficult to exclude; in fact four calli did have CAT activities that were higher than any of the negative controls (Figure 4.3a). It is difficult to say precisely how much greater the activity was in these calli as the assay is not quantitative, but by comparison with pCAP212 that expresses *cat* from the T-DNA 1' promoter it was very weak. It is possible that in these samples an elevated background activity was seen, so in the other assays (Figure 4.3b), calli of similar morphology were selected for the assay, and an estimate of protein content in each sample was performed. In none of the samples with the mitochondrial vectors was there activity above that of the controls. It was concluded that if the mitochondrial transformation vectors were expressing *cat* in these transformants, they were doing so at a level below the

Table 4.2

CAT Activity Detected in Calli Derived from Cotransformation of Mitochondrial and Nuclear Transformation Vectors.

Plasmid Combination	Number of Calli Assayed	Number >pLGV1103	Number ≤pLGV1103
pLGV1103	9	0	9
pLGV1103+pUPS92E	6	0	6
pLGV1103+pSCOX920	7	0	7
pLGV1103+pUPS92J	19	4	15
pLGV1103+pCAP212	16	10	6

The second column gives the number of calli tested after transformation with the plasmid combinations shown in the first column. Subsequent columns show the number which displayed CAT activity greater than, and less than or equal to that of the pLGV1103 transformants. pLGV1103 expresses only the NPTII gene; pCAP212 normally expresses the CAT and NPTII genes in the nucleus, however its NPTII gene was inactivated by restriction endonuclease digestion; the remaining plasmids are mitochondrial transformation vectors (Chapter 3).

Figure 4.3

Chloramphenicol Acetyl Transferase Activity in Kanamycin Resistant Calli Derived from Cotransformation with pLGV1103 and either pCAP212 or the Mitochondrial Transformation Vectors.

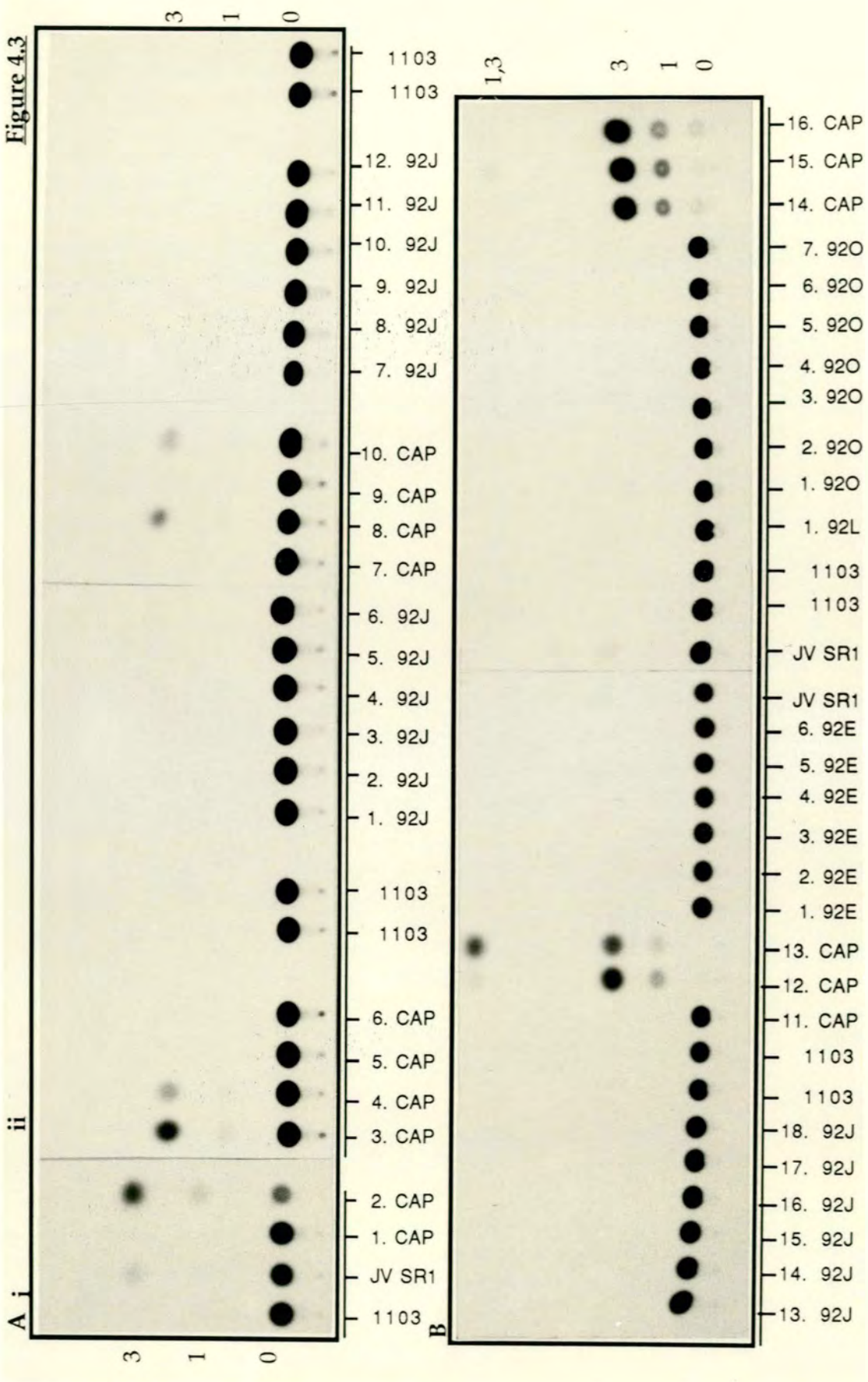
Protoplasts of *N. tabacum* cv. Petit Havana SR1 were transformed with linearised plasmid DNA of pLGV1103 that had been mixed with linear forms of plasmids pCAP212, pUPS92J, pUPS92E, or pSCOX92O. Protoplasts were also transformed with pLGV1103 DNA alone. The PEG-calcium nitrate transformation procedure was used and kanamycin resistant calli were recovered from agarose beads. CAT assays were performed as described in Materials and Methods on samples of about 50 mg of callus that had been grown on Murashige and Skoog medium supplemented with 50 µg kanamycin per ml. 0, 1, 3, and 1,3; chloramphenicol and its mono- and diacetylated forms respectively.

Part **A**, section **ii** shows the CAT activity demonstrated by twelve independent kanamycin resistant calli obtained from transformations using mixtures of pLGV1103 and pUPS92J (92J, numbers 1 to 12). Sections **i** and **ii** show the CAT activity demonstrated by several controls: kanamycin resistant calli recovered from transformations with pLGV1103 alone (1103) indicate the background activity in the transformed tissue; leaf tissue from a plant stably transformed with pCAP212 which was known to express CAT activity was provided by Dr. H-H Steinbiß, Max Planck Institut Köln, and included as a positive control for the CAT assay (JVSR1); ten independent kanamycin resistant calli derived from transformations using mixtures of pLGV1103 and pCAP212 were assayed to provide evidence for cotransformation and to estimate its frequency (CAP, numbers 1 to 10).

Four of the twelve potential pUPS92J cotransformants (numbers 2, 4, 6, and 9) demonstrated CAT activity slightly greater than that of the pLGV1103 controls. Five of the ten potential pCAP212 cotransformants (numbers 2, 3, 4, 8, and 10) demonstrated levels of CAT activity considerably greater than any of the pLGV1103 controls or the potential pUPS92J cotransformants.

Part **B** is a similar analysis of a further six potential pUPS92J cotransformants (92J, 13 to 18), six potential pUPS92E cotransformants (92E, 1 to 6), and seven potential pSCOX92O cotransformants (92O, 1 to 7). 92L indicates a callus derived from cotransformation with a plasmid constructed similarly to pUPS92J. In addition, a further six potential pCAP212 cotransformants and four pLGV1103 transformants were assayed. Of all these calli, only five (pCAP212 cotransformants 12 to 16) demonstrated activity greater than the pLGV1103 controls.

Figure 4.3


A
i
ii
3
1
0
1103
1103
12. 92J
11. 92J
10. 92J
9. 92J
8. 92J
7. 92J
10. CAP
9. CAP
8. CAP
7. CAP
6. 92J
5. 92J
4. 92J
3. 92J
2. 92J
1. 92J
1103
1103
6. CAP
5. CAP
4. CAP
3. CAP
2. CAP
1. CAP
JV SR1
1103
B
1,3
3
1
0
16. CAP
15. CAP
14. CAP
7. 92O
6. 92O
5. 92O
4. 92O
3. 92O
2. 92O
1. 92O
1. 92L
1103
1103
JV SR1
JV SR1
6. 92E
5. 92E
4. 92E
3. 92E
2. 92E
1. 92E
13. CAP
12. CAP
11. CAP
1103
1103
18. 92J
17. 92J
16. 92J
15. 92J
14. 92J
13. 92J

limit of detection by the assay used.

Secondly, this experiment has shown that the mitochondrial transformation vectors express *cat* far less efficiently than pCAP212 which allows selection of resistant cells (Chapter 6). Therefore, proceeding to precisely quantify the CAT activity expressed in nuclear transformants was not as important as determining whether or not it would allow such calli to survive the selection conditions that would be used to obtain mitochondrial transformants. Subsequent observations suggested that it is unlikely that such calli would survive. All chloramphenicol resistant nuclear transformants recovered so far have been shown to contain CAT activities similar to the positive pCAP212 transformants tested above. It was also found that growth of the potential pUPS92J cotransformants was prevented by even the least stringent screen for chloramphenicol resistance that has yet been successfully employed (10 μg/ml chloramphenicol in solidified Murashige and Skoog Medium, chapter 6). In agreement with this, potential pCAP212 cotransformants expressing similar levels of activity as the potential pUPS92J cotransformants are also sensitive to chloramphenicol and indistinguishable from untransformed or pLGV1103 transformed calli (Chapter 6). Thus whatever the level of nuclear expression of *cat* from the mitochondrial transformation vectors, it appears insufficient to confer a selectable chloramphenicol resistant phenotype.

In the above, it has been assumed that the mitochondrial transformation vectors have been integrated in an expressible form in the genomes of many of the potential cotransformants analysed. This assumption has been supported by showing successful integration and expression of the *cat* gene of pCAP212 in 60% of the kanamycin resistant calli derived from parallel cotransformation experiments. However, it is possible that pCAP212 cotransforms far more efficiently than any of the other plasmids, which consequently may not be present in the potential cotransformants tested. This possibility was excluded by Southern hybridisation analysis which confirmed that the mitochondrial transformation vectors had become integrated into the genomic DNA of some of the potential cotransformants. But before describing this experiment, one potential source of bias in cotransformation which has not yet received attention in the literature, but which is of relevance to the experiment above, will be discussed. To this end, the discussion will briefly review what has been learned of the fate of nucleic acids transferred into plant cells, and the conclusions will be used to postulate potential mechanisms by which plant cells are transformed and cotransformed by DNA molecules.

It can be postulated that the limiting step in cotransformation is the physical linkage of the nonselected plasmid to the selected one prior to or during integration. If so, conditions that influence the efficiency with which particular pairs of plasmids become linked will also influence their frequency of

cotransformation. There is considerable but disparate published evidence to suggest that such linkage is important for cotransformation, and that different pairs of plasmids may in fact be linked with differing efficiencies.

Firstly, based on the frequency of single transformation events, (about 10^{-3}) cotransformation occurs so much more frequently than expected (the product of the single transformation frequencies, 10^{-6}) that there must be some mechanism or condition that promotes it.

Secondly, the initial entry of DNA molecules into the plant cell does not appear to be the major factor limiting transformation frequencies. Efficient transient expression has been achieved with a gene whose expression is dependent upon the presence and expression of two different plasmids within the same cell (Ma *et al.* 1988). Following electroporation with Cucumber mosaic Virus (CMV) RNA, 65 % of rice protoplasts and up to 95 % of tobacco protoplasts were shown to express the coat protein (Okada *et al.* 1988). The genome of CMV consists of three distinct molecules that are required in the same cell for infectivity and replication (Peden and Symonds 1973), and it appears that all three were present in many of the electroporated protoplasts as the RNAs were shown to have replicated. Rice protoplasts do not normally maintain Tobacco Mosaic Virus, but CMV clearly can be maintained; Okada *et al.* (1988) found that following electroporation with a mixture of the two viral genomes, 20% of rice protoplasts that stably maintain CMV also maintain Tobacco Mosaic Virus. Thus most cells in a population receive nucleic acid in a form in which it can be expressed, and many receive more than one nucleic acid molecule. In fact, a more extensive comparison of stable and transient transformation efficiencies for DNA and for RNA suggests that during transformation, although about 60% of plant cells usually receive and express DNA, only a small percentage of this DNA is stably integrated in functional form; this in turn occurs in probably only a small fraction, often less than 1%, of cells that receive DNA (Langridge *et al.* 1985, Nishiguchi *et al.* 1986, Hauptman *et al.* 1987, Pröls *et al.* 1988, Okada *et al.* 1988, Gallie *et al.* 1989). Thus it seems likely that entry of the DNA into the cell, and perhaps even the nucleus, does not limit stable transformation rates, but that integration of the DNA into a site in the nuclear genome may do so. There is some debate as to what extent the nuclear membrane presents a barrier to transformation. The observation that transformation efficiencies are increased if DNA is microinjected directly into the nucleus rather than the cytoplasm suggests that the nuclear membrane may present a considerable barrier (Reich *et al.* 1986, Crossway *et al.* 1986). Similarly, the absence of a nuclear membrane at metaphase has been invoked to explain the increased efficiencies of transformation that were observed when cells were synchronised in metaphase before transformation by the PEG-calcium nitrate procedure (Meyer *et al.* 1985). However, in the same study, similar transformation frequencies appear to have been obtained with

S-phase protoplasts which possess a complete nuclear membrane (Meyer *et al.* 1985). Additionally, transient expression is efficiently achieved with asynchronous cell populations and transcription of the transferred DNA appears to be completed before the nuclear membrane degenerates (Pröls *et al.* 1988 and Töpfer *et al.* 1988, Hauptmann *et al.* 1987); if transient expression occurs in the nucleus, as seems probable (see section 4.4.2 below), these observations also imply that the nuclear membrane may not be a significant barrier to integration of transforming DNA. Whatever the principal constraint, be it entry of a subset of transferred molecules into the nucleus, the genome, or both, the arguments below apply equally.

If there were a mechanism for linking DNA molecules together once inside the cell, either before or during integration, then cotransformation would clearly be more frequent because it would result from a single integration event, and not two independent ones. If such linkage was prevalent, it could be a major influence on cotransformation as initially postulated. In animal cells such linkage is known to occur. In particular, in mammalian cells the transforming DNA is linked into large concatemers and temporarily maintained, independently of the genome, prior to integration (Scangos and Ruddle 1981). With plant cells, Paszkowski *et al.* (1984), Hain *et al.* (1985), Czernilofsky *et al.* (1986a) and Riggs and Bates (1986) have all shown there to be extensive concatenations and rearrangements of the integrated DNA following a variety of DNA transfer techniques. Wirtz *et al.* (1987) have recently characterised at least part of this recombination process.

They found that upon transformation of tobacco protoplasts, two plasmids bearing different portions of the NPTII gene, recombined to generate an intact and functional copy of the gene which became integrated into the genome. This event occurred with a frequency of up to 30% of that obtained by transformation with the intact gene. As with recombination in mammalian cells, its frequency was maximal when one of the plasmids had a double strand break in a region that was homologous between the two. Paszkowski *et al.* (1988) have reported that recombination between transforming DNA and a homologous genomic sequence is promoted by a double strand break in the homologous region of the transforming DNA. Thus, there is a mechanism that could potentially influence the frequency with which specific pairs of plasmids are recombined and thereby linked. As postulated above, this could in turn influence the cotransformation frequencies obtained with different pairs of plasmids.

This potential source of bias in cotransformation frequencies is of particular relevance to the cotransformation experiment described above. Before transformation, the positive control, pCAP212, was digested at its two *Pst*I sites to generate double stranded breaks in the DNA. Both these sites lie within regions that are homologous over several hundred base pairs to pLGV1103 which was the

selectable plasmid in the experiment. In contrast, there is no such homology to pLGV1103 around the *Pvu*II sites at which the mitochondrial transformation vectors were digested. Thus the recombination process described above would link pLGV1103 to the mitochondrial transformation vectors less efficiently than to pCAP212, and their respective cotransformation efficiencies may be reduced as a result.

To what extent recombination mechanisms other than the one investigated by Wirtz *et al.* (1987) may contribute to linking of transferred DNA molecules is currently unknown. It is worth noting that following transformation by direct DNA transfer techniques, in nearly all cases that have been examined, several copies of the transforming DNA appear to be present at a single site of integration, or in one case at different but closely linked sites in the genome (Riggs and Bates 1986). It is possible that this linkage occurs before or during integration into the genome in a manner similar to that proposed above, but it also may be the result of several entirely independent integrations at the same site. Thus part of the unexpectedly high cotransformation efficiency may simply result from there being, in just a few cells at one time only a limited number of sites that are competent to integrate DNA. Only those cells in the population that possess such sites when transforming DNA is present would proceed to integrate it, and several different molecules may then be integrated independently at each site. If two different plasmid species are present, it is likely that one copy of each would be integrated in such a way that it could be expressed. The remaining cells would simply express the transferred DNA transiently, until it was lost, perhaps within the first hour after delivery (Pröls *et al.* 1988). Such competent sites may be sites of DNA replication, recombination, or repair. This proposed mechanism provides an explanation for the increased frequency of transformation observed by Meyer *et al.* (1985) using both S phase and metaphase cells.

Southern Hybridisation Analysis of the Potential Cotransformants.

Currently it is unclear to what extent each of these processes may contribute to the final cotransformation efficiency, or whether here is a bias in cotransformation rates. Therefore it was important to show by Southern Blotting that some of the potential cotransformants did contain integrated forms of the mitochondrial transformation vectors in their genomes.

Total cellular DNA was extracted from two potential pUPS92J cotransformants and two potential pCAP212 cotransformants. The former were digested with *Hae*III and with *Pvu*II, and the latter with *Pvu*II and *Hin*dIII. DNA from a plant transformed with pLGV2103 (Czernilofsky *et al.* 1986a) which is a

partial dimer of pLGV1103, was used as a negative control to eliminate the possibility that hybridisation was to this plasmid. The digestion products were separated by agarose gel electrophoresis, blotted onto nitrocellulose and probed with the 1050 bp *Hae*III fragment from pUPS92J containing 50 bp of *coxI* , the entire CAT gene and 200 bp beyond the polylinker cloning site (Figures 3.14 and 4.4A, Yanisch-Perron *et al.* 1985)

Figure 4.4A shows that hybridisation is observed to several fragments in DNA from pUPS92J cotransformant number 16, the major *Hae*III fragment appearing slightly larger than 1 kb as expected. The pUPS92J plasmid DNA used in the initial transformation had been digested with *Pvu*II; the *Pvu*II fragment with most homology to the probe is cut by *Hae*III to produce a single homologous fragment of about 1 kb (Figures 3.14 and 4.4A). The hybridising fragment in plant genomic DNA will be larger because the second *Hae*III site will lie at some point beyond the insert in the plant genomic sequence. The other hybridising bands may result from integration of other copies of the insert either in a modified form, or with different flanking sequences, or may result from hybridisation to the other *Pvu*II fragment in the transforming DNA which has 18 bp of homology to the probe. This fragment also may have been modified. As discussed above such modifications to the DNA are common. The *Pvu*II digest shows hybridisation to high molecular weight DNA as expected because the homologous fragments have no internal *Pvu*II sites. This enzyme is expected to cut plant DNA poorly as it is sensitive to the cytosine methylation that occurs in plant cells (Hepburn *et al.* 1987). There was no significant hybridisation to genomic DNA of any of the other transformants. The CAT gene of pCAP212 shares only 73% homology to *cat* of pUPS92J, and was not expected to detectably hybridise with the probe (Charles *et al.* 1985b, Manniatis *et al.* 1982).

Figure 4.4A

Southern Blot Hybridisation Analysis of Potential Cotransformants.

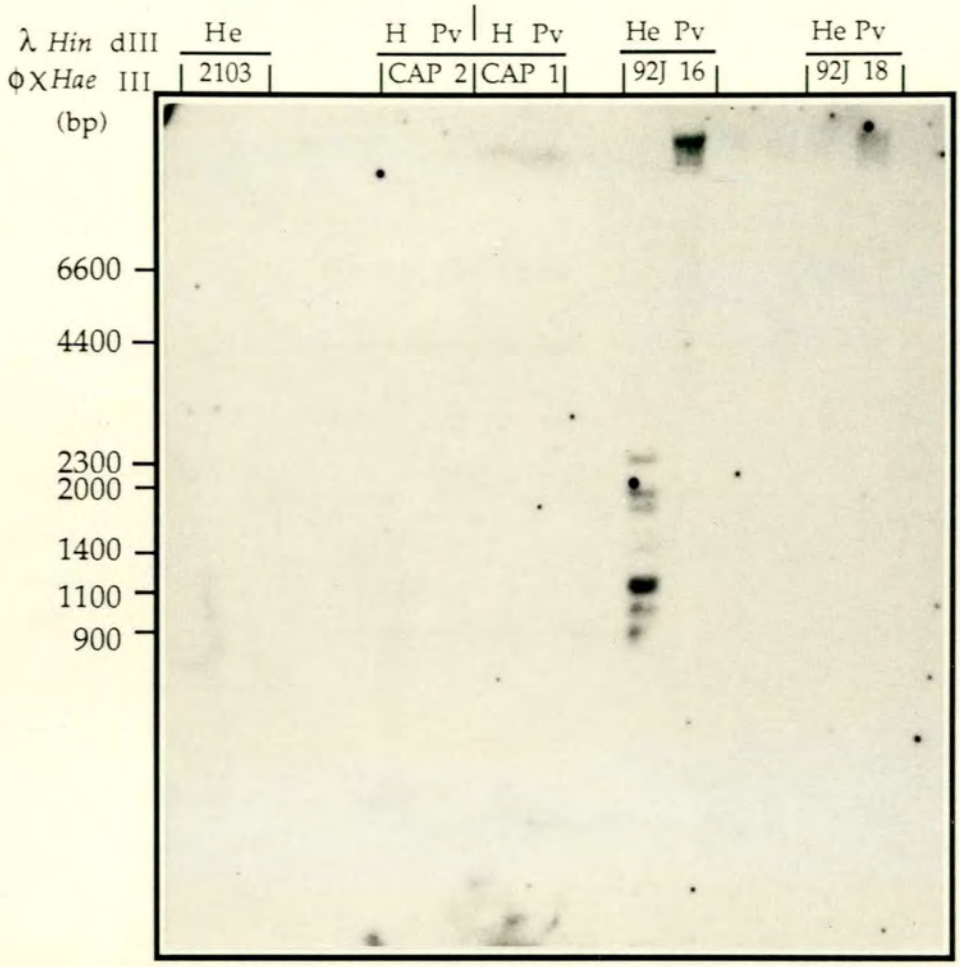
Total genomic DNA was isolated from kanamycin resistant transformants obtained by transformation of protoplasts with mixtures of pLGV1103 and either pUPS92J or pCAP212. Between 2 and 4g of callus from clones designated 92J16, 92J18, CAP1 and CAP2 in Figure 4.3 were used for DNA isolation according to the CTAB method described in Materials and Methods. Genomic DNA isolated from leaves of a plant transformed with plasmid pLGV2103, which is a partial dimer of pLGV1103 was provided by Dr. A.P. Czernilofsky Max Planck Institut, Köln. Approximately 10 μg of genomic DNA was digested with either *Hae*III (He), *Hin*dIII (H) or *Pvu*II (Pv) and the digestion products were separated by agarose gel electrophoresis in TEA buffer at 40 V for 24 hours as described in Materials and Methods. The DNA was transferred to nitrocellulose by capillary blotting for 12 hours, and the filter was baked, prehybridised and hybridised with a nick-translated probe and washed as described in Materials and Methods. The filter was exposed to Kodak X-ray film for 7 days at -70^{0}C with intensifying screens.

The probe was derived from the 1050 bp *Hae*III fragment of pUPS92J. Its origin in pUPS92J and its known sequence homology with the *Pvu*II digestion products of this plasmid are indicated below the autoradiopraph. Plasmid pUPS92J had been digested with *Pvu*II prior to protoplast transformation.

Strong hybridisation was observed to genomic DNA from only 92J16, though there may be weak hybridisation to the *Pvu*II digest of DNA from 92J18. Ethidium bromide staining of the agarose gel revealed that less DNA from 92J18 had been loaded than from the other samples (Figure 4.4B), and this may have contributed to the lower intensity of hybridisation observed. Hybridisation was not observed to genomic DNA from any of the remaining samples indicating that the probe had not hybridised to the pLGV1103 sequences.

The top of the autoradiograph corresponds to the wells of the agarose gel. The sizes of a *Hin*dIII digest of bacteriophage lambda DNA and a *Hae*III digest of bacteriophage ϕX174 RF DNA that were used as DNA size markers are indicated to the left.

Figure 4.4 A


λ Hin dIII
ϕX Hae III
(bp)
He
2103
H Pv
CAP 2
H Pv
CAP 1
He Pv
92J 16
He Pv
92J 18
6600
4400
2300
2000
1400
1100
900

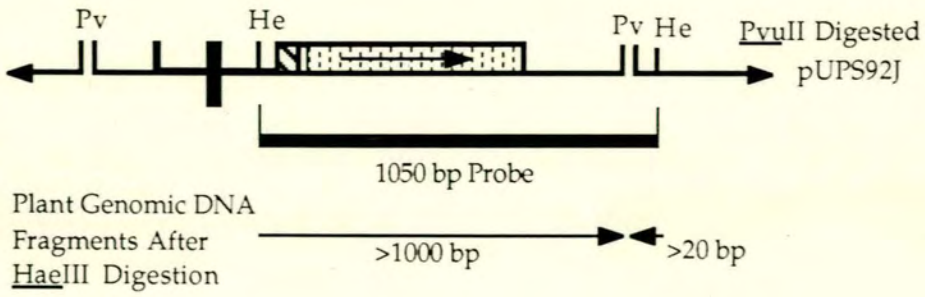

Pv
He
Pv He
PvuII Digested
pUPS92J
1050 bp Probe
Plant Genomic DNA
Fragments After
HaeIII Digestion
>1000 bp
>20 bp

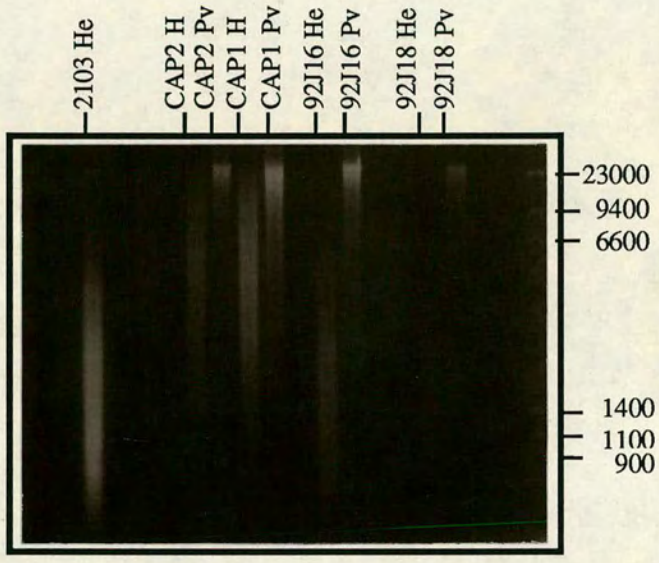
Figure 4.4B

Southern Blot Hybridisation Analysis of Potential Cotransformants.

Total genomic DNA was isolated from kanamycin resistant transformants obtained by transformation of protoplasts with mixtures of pLGV1103 and either pUPS92J or pCAP212. Between 2 and 4g of callus from clones designated 92J16, 92J18, CAP1 and CAP2 in Figure 4.3 were used for DNA isolation according to the CTAB method described in Materials and Methods. Genomic DNA isolated from leaves of a plant transformed with plasmid pLGV2103, which is a partial dimer of pLGV1103 was provided by Dr. A.P. Czernilofsky Max Planck Institut, Köln. Approximately 10 μg of genomic DNA was digested with either *Hae*III (He), *Hin*dIII (H) or *Pvu*II (Pv) and the digestion products were separated by agarose gel electrophoresis in TEA buffer at 40 V for 24 hours as described in Materials and Methods. The DNA was stained with ethidium bromide and photographed under U.V. illumination.

The top of the photograph corresponds to the wells of the agarose gel. The sizes of a *Hin*dIII digest of bacteriophage lambda DNA and a *Hae*III digest of bacteriophage ϕX174 RF DNA that were used as markers are indicated to the right.

Figure 4.4 B


2103 He
CAP2 H
CAP2 Pv
CAP1 H
CAP1 Pv
92J16 He
92J16 Pv
92J18 He
92J18 Pv
23000
9400
6600
1400
1100
900

4.4.2 Transient Expression Analysis with the Mitochondrial Transformation Vectors.

The experiments outlined above are useful in that they directly assay the expression of *cat* from mitochondrial transformation vectors that are known to be stably integrated into the nuclear genome. None-the-less, they have several disadvantages. Firstly, it is laborious to prove that the mitochondrial transformation vectors have been integrated into genomic DNA. Secondly, although Southern hybridisation analysis can show the presence of these vectors, it cannot be shown that they have been integrated in a potentially expressible form; this must be assumed by analogy with expression of *cat* in pCAP212 cotransformants. Thirdly, variation in the low levels of CAT activity seen with potential cotransformants and negative controls is difficult to interpret. This variation may be due in part to the presence or absence of expressible mitochondrial vector sequences, but also to variation in background activity between individual calli and to the variation that is usually observed in independent transformants in the levels of expression from transferred DNA (Paszkowski *et al.*1984, Shillito *et al.*1985, Czernilofsky *et al.*1986b, Dean *et al.* 1987)

Analysis of transient expression in protoplasts provides a more rapid method for investigating the expression of genes in plasmid constructs, and one that avoids the problems listed above. The limitation of this technique is that whilst nuclear genes are often efficiently expressed, it is unclear what can be inferred about the activity that would result from the same genes if they were stably integrated into the genones of callus or differentiated cells. This is partly because little is known of the mechanism or circumstances of transient expression in plant cells. Nishiguchi *et al.* (1986) and Okada *et al.* (1988) found that following transfer to protoplasts viral RNA was detectably expressed in about 70% of them, and Pröls *et al.* (1988) found that transcription and translation of a chimaeric CAT gene was complete within the first few hours after transfer. It is not known whether transcription is occurring in the nucleus. Successful transient expression from transferred mRNA is dependent upon the presence of a 5' ^{m7}Gppp cap structure and 3' polyadenylation (Callis *et al.* 1987a), implying that the transferred nucleic acid is not confined to the nucleus. However, the observation that plasmids containing introns can also be efficiently expressed, and that the introns can even enhance transient expression perhaps strengthens the argument for nuclear transcription (Callis *et al.* 1987b).

Transient expression has been achieved with very many different nuclear gene constructs and mRNAs. The nucleotide sequence requirements and the inhibitor sensitivity of transient expression are the same as those of typical nuclear gene expression, so the same gene expression mechanisms appear to

operate in each case (for example Callis *et al.* 1987a and b, Pröls *et al.* 1988, Ma *et al.* 1988). Thus, it is reasonable to argue that if *cat* in the mitochondrial transformation vectors could be constitutively expressed in tobacco nuclei, such expression would be revealed in transient expression experiments.

4.4.2.1 Establishment of a Transient Expression System with Tobacco Protoplasts.

Transient expression in protoplasts isolated from the leaves, hypocotyls, roots and cultured cells of a variety of monocots and dicots has been reported (Junker *et al.* 1987, Fromm *et al.* 1985, Hauptmann *et al.* 1987). The most thoroughly studied and frequently used techniques have involved electroporation and PEG-induced transformation of tobacco mesophyll protoplasts.

Initially, successful transient expression of several nuclear gene constructs in mesophyll protoplasts was achieved using the PEG-calcium nitrate procedure of Hein *et al.* (1982) and Pröls *et al.* (1988), but later it became clear that there was a level of variability in the system that could lead to qualitatively different results when two plasmids were compared on separate occasions (discussed in section 4.5). The PEG-calcium nitrate transformation procedure is complex and usually lethal to between 50 and 70% of the protoplasts. In my experience, the survival of the protoplasts could be greatly affected by the way they were mixed with each of the solutions during transformation, and this seemed a likely source of variability. In contrast, electroporation involves few manipulations, and so was evaluated as an alternative transfer technique, and as a way of eliminating some of the variability of the system.

At the same time, a tobacco suspension culture was developed as an alternative source of protoplasts because in general such cultures are convenient to maintain, give high yields of viable protoplasts and may be a better target for mitochondrial transformation than mesophyll protoplasts because the cells and presumably the mitochondria are actively dividing (Uchimaya and Murashige 1974, Fromm *et al.* 1985). Suspension derived protoplasts were then used to evaluate electroporation.

Isolation of Protoplasts from Suspension Culture Cells.

Protoplasts were isolated from a suspension culture of *N. tabacum* cv.

Xanthi using a protocol developed from Uchimaya and Murashige (1974). Firstly the growth of the culture and the optimal time after subculture for protoplast isolation were investigated. A culture was grown for three weeks, being diluted four fold into fresh medium each week, and protoplasts were isolated at daily intervals during the fourth week. Cell walls of cells from 10 ml aliquots of culture were removed by a five hour enzymatic digestion as described in materials and methods. Then without further purification, the number of protoplasts and the total number of cells were estimated by directly counting an aliquot of the digestion mixture in a haemocytometer. Protoplasts were identified as being spherical and separate from other cells. Each sample was incubated for a further 16 hours without shaking and then recounted, but no change in the yield of protoplasts was observed.

Figure 4.5 shows that the fresh weight of cells recovered from 10 ml aliquots of the culture increases dramatically between days 2 and 4 accompanied by a less pronounced increase in total cell number and protoplast yield. The efficiency of protoplast isolation, measured as percentage of total cells, and the absolute yield of protoplasts increases steadily to the optimum at day 4, declining rapidly thereafter. At this stage, most of the digestion products are misshapen and do not qualify as protoplasts on the criteria used above, although these cells may still be competent for transient expression. These results are similar to those reported by Uchimaya and Murashige (1974) except that their culture showed a longer lag phase, perhaps because it was subcultured fortnightly, and not weekly. On the basis of these results, cultures were subcultured every 4 or 5 days and protoplasts isolated 3 or 4 days later.

Preliminary experiments using a nuclear CAT expression vector pRT-T1 (section 6.4) showed that suspension derived protoplasts isolated 3 or 4 days after subculture were viable for transient expression using the PEG-calcium nitrate procedure (figure 4.6A). A transformation experiment was also performed with the misshapen digestion products isolated from 5 day old cultures, but no evidence of expression was found, and the use of cultures older than 4 days was not pursued. Suspension derived protoplasts were then used to evaluate electroporation.

Electroporation of Suspension Derived Protoplasts.

The conditions used for electroporation are given in Materials and Methods. The only parameter that was investigated was the optimal field strength for the equipment and protoplasts used in this work. Pulses of relatively low voltage and long duration were employed as recommended by Fromm *et al.* (1985). Twelve

Figure 4.5

Determination of the Optimal Time After Subculture for Isolation of Protoplasts from Suspension Culture Cells.

A suspension culture of *N. tabacum* cv. Xanthi was grown for three weeks, being diluted four fold into fresh medium each week, and protoplasts were isolated at daily intervals during the fourth week. Cell walls of cells from 10 ml aliquots of 0 to three day old culture and 5 ml aliquots of four to seven day old culture were removed by a five hour enzymatic digestion as described in Materials and Methods. Then without further purification, the number of protoplasts and the total number of cells were estimated by directly counting an aliquot of the digestion mixture in a haemocytometer. Each sample was counted four times and the final value is an average of these. Values are normalised for 10 ml of culture. Protoplasts were identified as being spherical and separate from other cells. Each sample was incubated for a further 16 hours without shaking and then recounted, but no change in the yield of protoplasts was observed.

The upper graph shows the weight of cells that could be recovered from 10 ml of culture on each day. Each aliquot was centrifuged at about 100 xg for 10 minutes in a preweighed tube, the medium was pipetted off and the tube was reweighed to determine the packed cell weight.

The centre graph shows the number of protoplasts estimated in each sample after digestion of cell walls as described above, and also the total number of cells (including protoplasts) that remained in the sample. These numbers were used to calculate the percentage of surviving cells that had been converted to protoplasts, and this is shown in the lower graph.

The optimal time for isolation of protoplasts appeared to be four days after subculture.

Figure 4.5

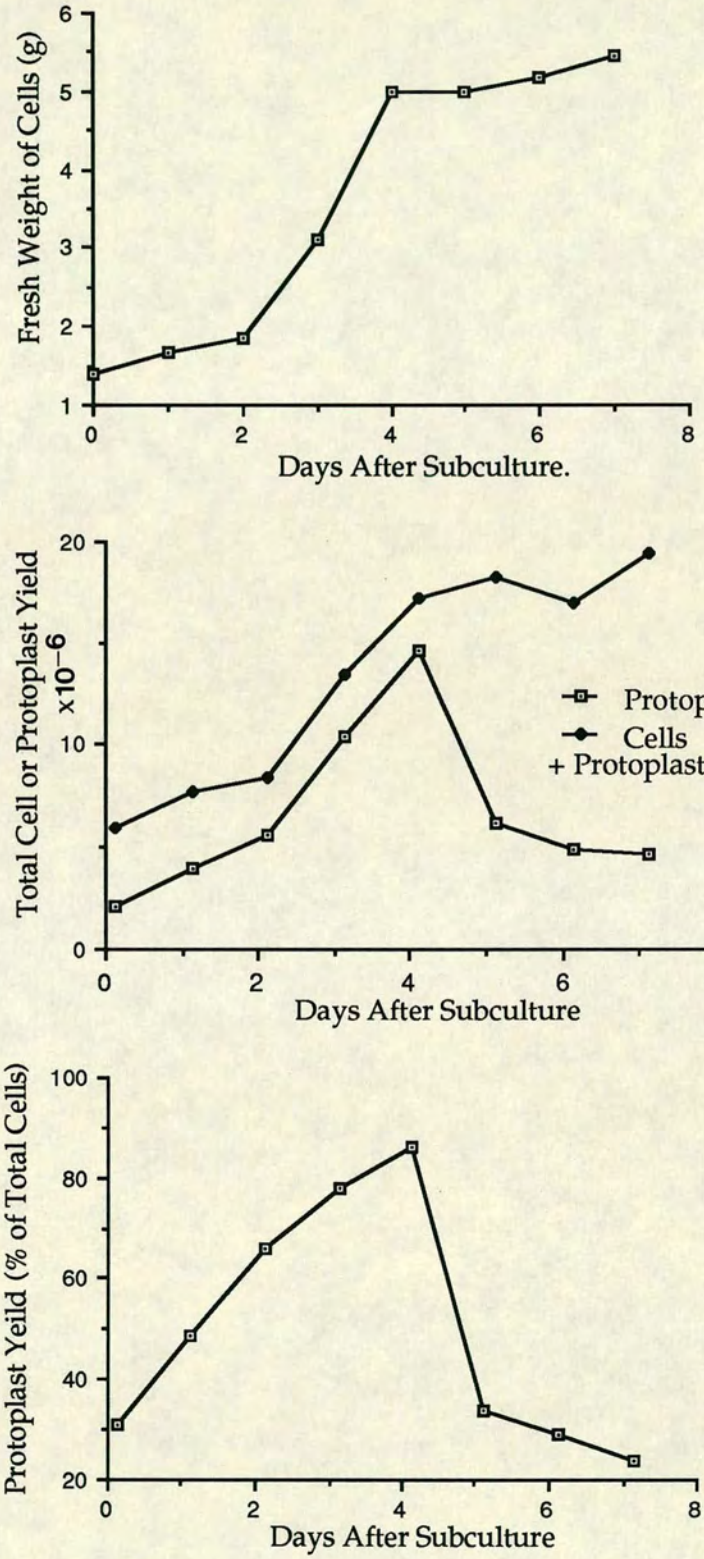

Fresh Weight of Cells (g)
Days After Subculture.
Total Cell or Protoplast Yield x10−6
Protoplasts
Cells + Protoplasts
Days After Subculture
Protoplast Yeild (% of Total Cells)
Days After Subculture

Figure 4.6 A

Transient Expression in Suspension Culture Derived Protoplasts.

Five million protoplasts were isolated from suspension cultures as described in Materials and Methods, and divided into three aliquots. The PEG-calcium nitrate transformation procedure was used to transform one sample with either 40 μg of supercoiled DNA of plasmid pRT-T1, 40 μg of supercoiled plasmid p92J-S13 or with LTE buffer lacking DNA. Plasmid pRT-T1 is described in section 6.4, and expresses CAT in plant nuclei; plasmid p92J-S13 is described in section 5.2.1 and Figure 5.4, and is a derivative of the mitochondrial transformation vector pUPS92J that additionally contains DNA sequence derived from the tobacco mitochondrial genome. Transformation and the CAT assay were performed as described in materials and methods. Approximately 50 mg of callus from a potential mitochondrial transformant designated 920-S13#1 (section 6.6) was included in the CAT assay. ^{14}C indicates a sample of the radioactive chloramphenicol that was used to assay the CAT activity in the other samples.

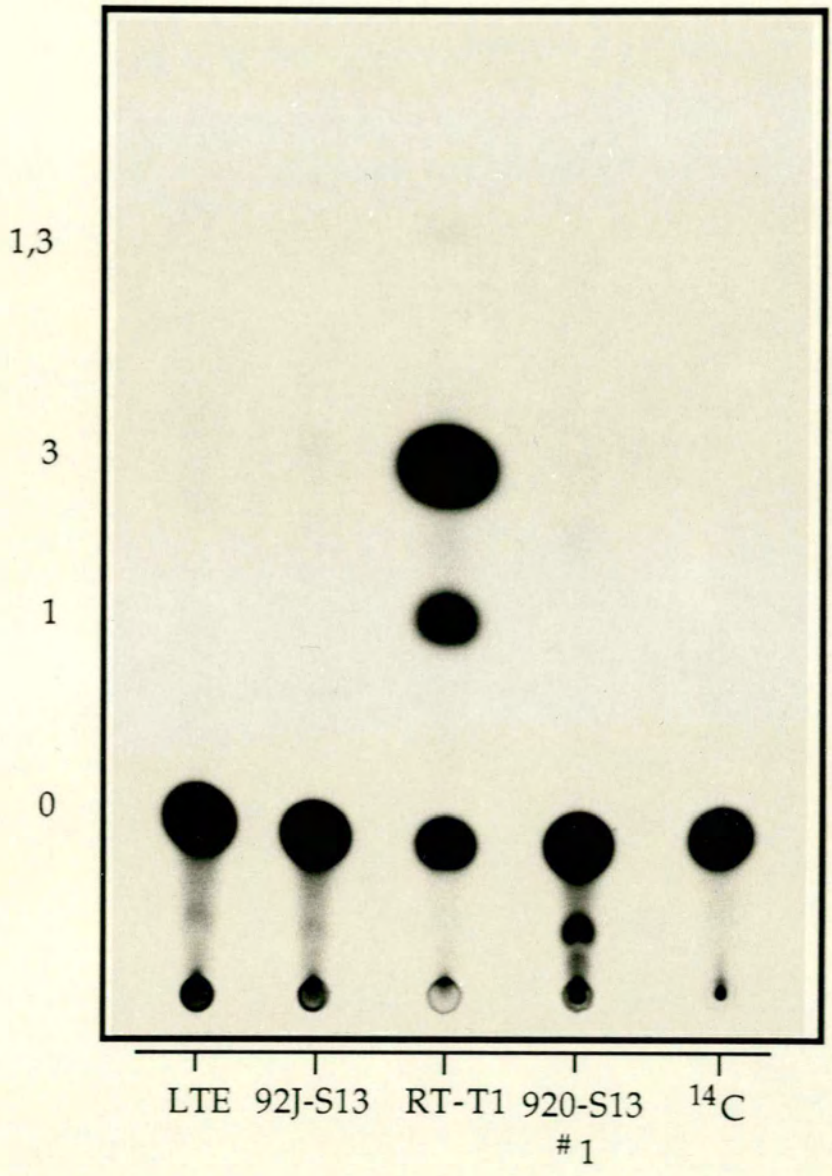
0, 1, 3, and 1,3 indicate the positions of chloramphenicol, its two monoacetylated forms and the diacetylated form respectively.

CAT activity was detectable in the protoplasts transformed with pRT-T1 indicating that the protoplasts are viable for transient expression.

CAT activity in the sample transformed with p92J-S13 was not greater than in the sample transformed with buffer alone suggesting that the CAT gene in this plasmid was not expressed (section 4.4.2.2). Similarly, CAT activity could not be detected in the tissue from 920-S13#1.

The apparently acetylated forms of chloramphenicol observed in the latter three samples probably arise from a background activity in tobacco tissue as they are absent from the radioactive chloramphenicol preparation added to the sample.

Figure 4.6 A



million protoplasts were isolated from 6 g of packed suspension culture, and divided into eleven samples. About 5 µg of pRT-T1 which expresses the Type I CAT gene (see section 6.4 and Figure 6.6B) was added to ten of these, and electroporation was performed on each at a field strength between 0 V/cm and 1 kV/cm. One sample was electroporated without DNA at 400 V/cm which was the optimum deduced by Fromm *et al.* (1985).

An optimum was observed between 500 and 700 V/cm, but activity persisted up to 1 KV/cm and appeared to be DNA dependent (Figure 4.6B i). To confirm that an intrinsic CAT activity was not induced in protoplasts at field strengths above 400 V/cm the experiment was repeated without DNA (Figure 4.6B ii), and no evidence for such induction was found. The optimum field strength in this system is approximately 500 V/cm which is higher than that reported by Fromm *et al.* (1985), perhaps because they used a larger distance between the electrodes and consequently to generate the same field strength would have required higher electrode voltages which produce increased heating and damage to the protoplasts.

Using this technique experiments were performed to compare transient expression from five different plasmids, however the results obtained were disappointing. As shown in Figure 4.6B iii there was considerable variability between experiments in the activity displayed by protoplasts electroporated with the same plasmid. This variability was greater than that observed when using the PEG-calcium nitrate procedure (for example see Figure 4.11).

The relative CAT activities expressed by each construct were estimated by scanning the autoradiographs of the three duplicate assays using a Quick Scan R&D densitometer (Helena Laboratories), subtracting the background activity and summing the remainders for each plasmid. The sum of the CAT activities expressed from plasmids pRTUPS600, pRTBamFS, and pRTClaFS in the three duplicate electroporation assays produced relative activities of 100%, 50% and 75% respectively. These summed results resemble those obtained with the PEG-calcium nitrate technique (90%, 25%, and 100% respectively, Figure 4.11), though such similarity is not apparent from the individual assays themselves. Work has been initiated to locate the source of the variation either to the transformation procedure or to the assay, and although the electroporation system has not been fully optimised, it was decided that the PEG-calcium nitrate procedure was the method of choice.

Figure 4.6 B

Electroporation of Suspension Derived Protoplasts.

i. $1.2x10^7$ protoplasts were prepared from 6g of suspension culture cells and divided into eleven samples. Supercoiled DNA of plasmid pRT-T1 (a vector that is designed to express *cat* in plant cell nuclei Figure 6.6B) was added to ten samples and each was subjected to electroporation at electric field strengths between 0 and 1000 V/cm. The capacitance of the pulse generator was set to 25 μF, and the half life of the pulses ranged from 7.7 to 11.3 milliseconds. As a negative control, one sample was electroporated at 400 V/cm but no DNA was included. The CAT activity of each sample was assayed 26 hours later and the result is shown; 212 shows the activity in a chloramphenicol resistant callus transformed with pCAP212 that expresses *cat* . The optimum field strength for electroporation appears to be between 500 and 700 V/cm.

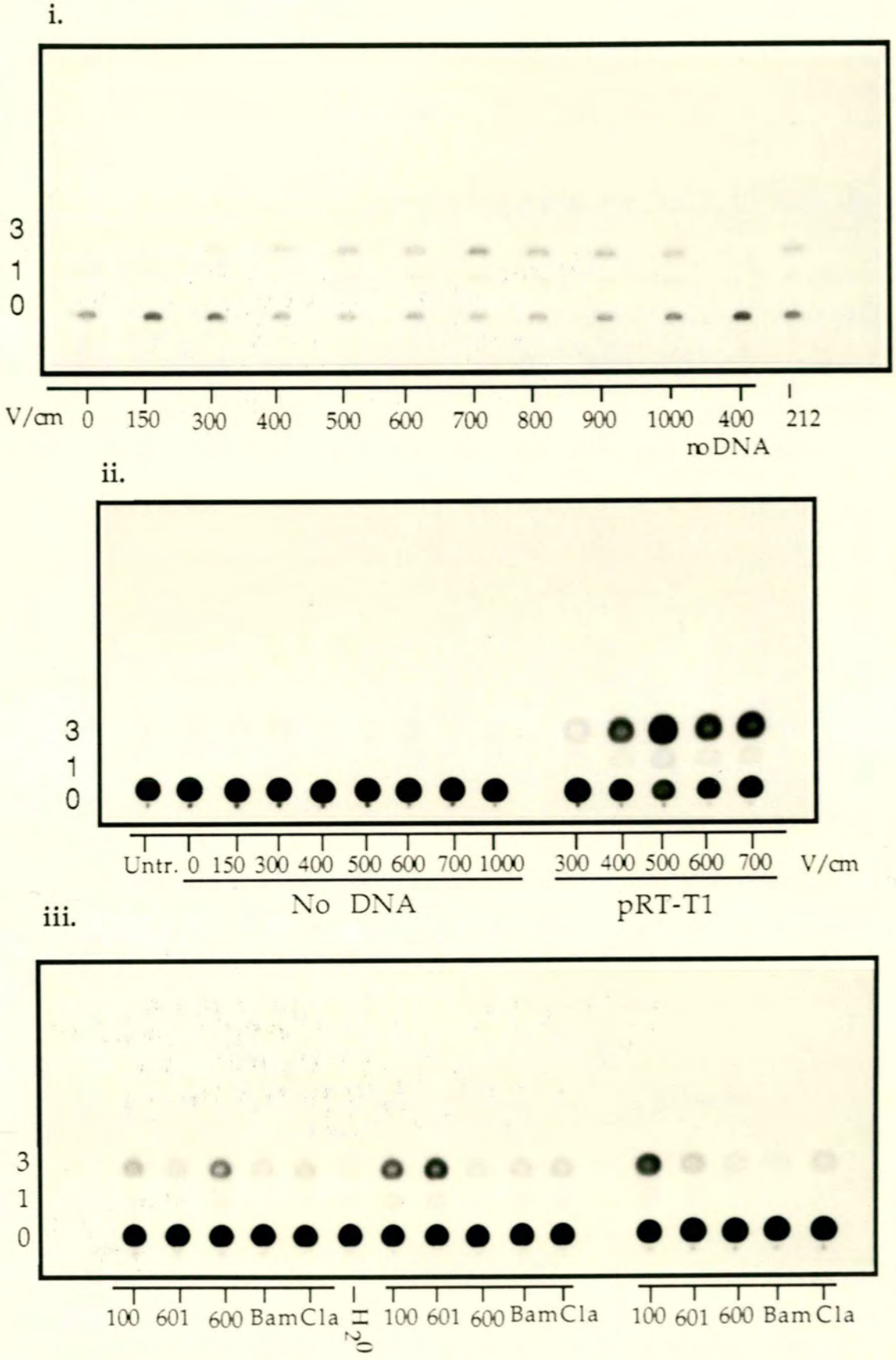
ii. Electroporation was performed at field strengths between 0 and 1000 V/cm without addition of DNA. $2x10^7$ protoplasts were prepared from 7g of 3 day old suspension culture cells and one million were used in each electroporated sample. The half life of the pulses ranged from 8 to 24 milliseconds. In addition 10 μg of supercoiled pRT-T1 DNA was added to five samples and these were subjected to electroporation at between 300 and 700 V/cm to confirm the result in section i. The half lives of the pulses ranged from 9.4 to 13.6 milliseconds. The CAT activity in each protoplast sample and in one plated in culture medium immediately after isolation (untr) were determined and are shown. Electroporation appears not to induce CAT activity in protoplasts in the absence of DNA. Electroporation in this system was optimal at 500 V/cm.

iii. This figure shows the CAT activity in protoplasts electroporated in the presence of 40 μg of supercoiled DNA of plasmids pRTCAT100 (100), pRTUPS600 (600), pRTΔ601 (601), pRTBamFS (BamFS) and pRTClaFS (ClaFS) that each express *cat* in plant cells at different levels. They are described in detail in section 4.5 and in Figures 4.8, 4.9A and B. The eleven samples at the left are derived from duplicate electroporation experiments performed with each plasmid on the same day and with aliquots of the same protoplast and DNA preparations. H_2O shows a sample in which water was added in place of DNA. The five remaining samples are derived from a second electroporation experiment performed with a different protoplast preparation but with the same DNA preparations.

The relative CAT activities obtained by densitometry of the autoradiographs were 270%, 175%, 100%, 50%, 75% and 0 for 100, 601, 600, BamFS, ClaFS and H_20 respectively.

0, 1 and 3 show positions of chloramphenicol and its monoacetylated forms.

Figure 4.6 B


i.
3
1
0
V/cm 0 150 300 400 500 600 700 800 900 1000 400 212
no DNA
ii.
3
1
0
Untr. 0 150 300 400 500 600 700 1000 300 400 500 600 700 V/cm
No DNA
pRT-T1
iii.
3
1
0
100 601 600 Bam Cla H2O 100 601 600 Bam Cla 100 601 600 Bam Cla

4.4.2.2 Testing the Mitochondrial Transformation Vectors.

Several different constructs have been assayed for transient expression of *cat* in both mesophyll and suspension derived protoplasts using both electroporation and the PEG-calcium nitrate transformation procedure. Plasmid pAPcat1 which was not included in the cotransformation experiment above was included in this analysis. Plasmids pRTCAT100, pRTUPS600 and pRT-T1 that express the *P. mirabilis* or Type I CAT genes in plant cell nuclei were used as positive controls. Although *cat* was expressed from the positive controls in these experiments, no activity was detected from any of the mitochondrial transformation vectors. As described in Chapter 5 derivatives of these basic vectors have been constructed. These derivatives contain additional sequences from the mitochondrial ATP9 gene and flanking sequence of either tobacco or *Petunia* which are intended to provide transcription termination signals and sequence homology for recombination with tobacco mtDNA. Transient expression analysis was also performed with several of these derivatives, and similarly CAT activity was not detected. Examples of these assays are shown in Figures 4.6A and 4.7.

Two important conclusions can be drawn. Firstly, it appears that *cat* cannot be expressed from any of the mitochondrial transformation vectors by the cellular mechanisms that are normally active on nuclear genes. This bodes well for their use in mitochondrial transformation experiments. Secondly, as no expression is detected at all, either transforming DNA is brought into contact with the mitochondrial gene expression system far less frequently than with the nuclear one, or alternatively if transforming DNA is equally available to each system during the assay period, far less CAT activity is expressed from these plasmids by the mitochondrial system than from nuclear expression markers by the nuclear one. If the former alternative is true, unless transferred DNA is far more efficiently integrated into mtDNA than into nuclear DNA, mitochondrial transformation using this delivery system will probably be rare compared to nuclear transformation. Transformation of yeast mitochondria was found to be less frequent than nuclear transformation (Johnston *et al.* 1988). If the latter alternative is true, the mitochondrially located CAT genes may have to become amplified several fold in each cell before the CAT activity and chloramphenicol resistance that results is equal to that conferred by a nuclear gene. As discussed in Chapters 7 and 8, these observations have serious implications for the efficiency of the transformation and selection procedures that will be required to isolate transformants.

Figure 4.7

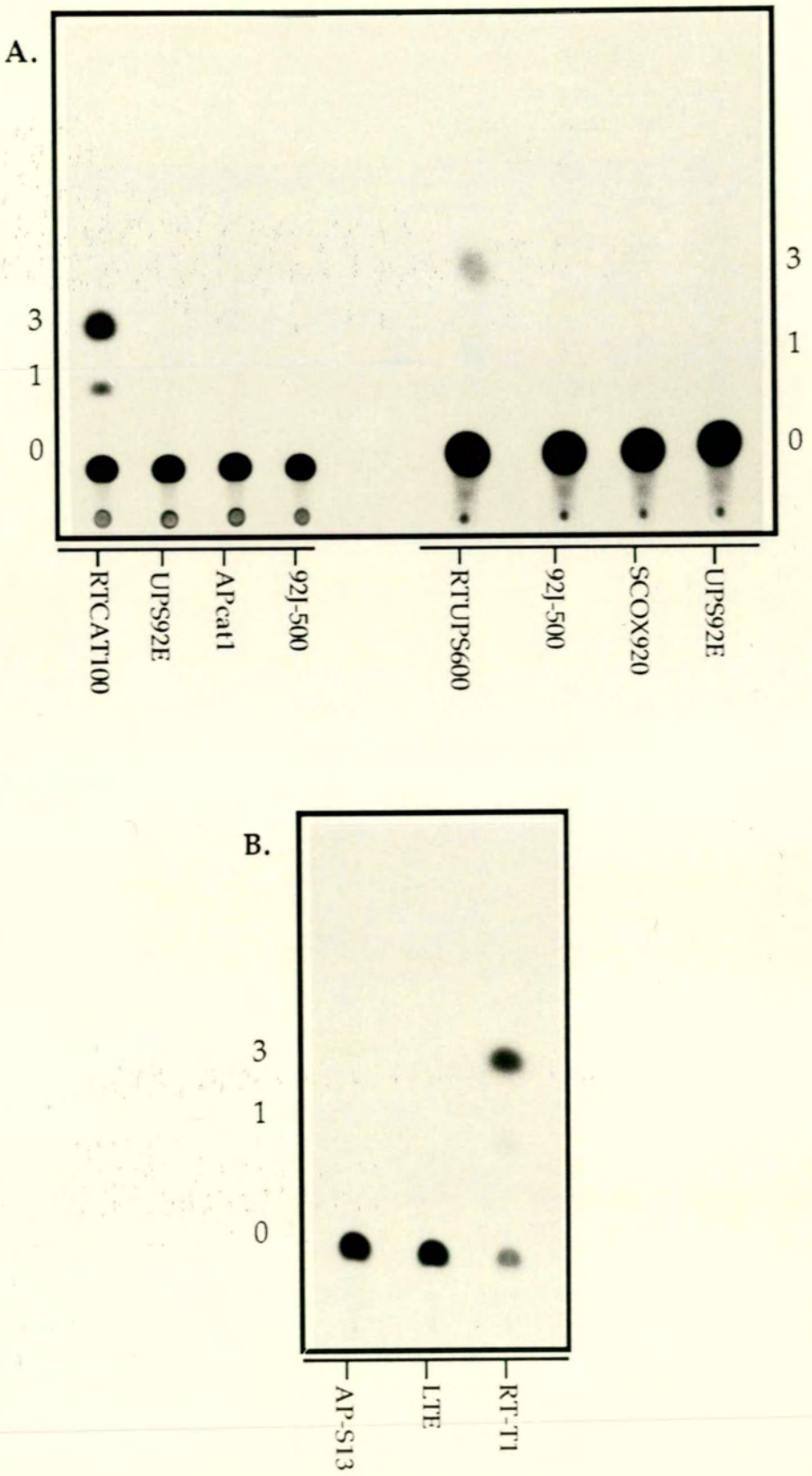
Transient Expression with the Mitochondrial Transformation Vectors.

A. Transient expression was performed using the PEG-calcium nitrate transformation procedure with leaf mesophyll protoplasts isolated from *N. tabacum* cv. Petit Havana SR1 as described in Materials and Methods. Approximately $1x10^6$ protoplasts and 40 µg of supercoiled plasmid DNA were included in each sample. As positive controls plasmids pRTCAT100 and pRTUPS600 were used; these are designed to express *cat* in plant cell nuclei (Figures 4.8 and 4.9A). The mitochondrial transformation vectors pSCOX920 and pAPcat1 which were described in sections 3.2.1 B and 3.2.2 were included in this analysis. Plasmid pUPS92E which contains the maize COXI gene promoter region inverted relative to the CAT gene was included as a negative control. Plasmid p92J-500 has not been described previously. This plasmid is a derivative of pUPS92J (Figure 3.9) which additionally contains the *Ava*II to *Pst*I fragment of the *P. hybrida* ATP9-1 gene promoter sequence (Figure 3.14) inserted using the *Eco*RI site upstream of the *coxI* promoter region of pUPS92J (not shown). CAT activities in samples transformed with pUPS92E were at levels expected for the background activity in tobacco protoplasts. Only those samples transformed with pRTCAT100 or pRTUPS600 demonstrated elevated CAT activity.

B. Transient expression of pAP-S13 was assayed in electroporated suspension culture derived protoplasts of *N. tabacum* cv. Xanthi. Plasmid pAP-S13 is described in Figure 5.6A and is a derivative of pAPcat1 that contains 2.9 kb of additional sequence from the tobacco mitochondrial genome. Protoplsts were mixed with either i) 40 µg of supercoiled DNA of pRT-T1 which is designed to express the Type I CAT gene in plant cell nuclei (Figure 6.6B), ii) 40 µg of supercoiled plus 40 µg of linear pAP-S13, or iii) buffer lacking DNA (LTE). The sample electroporated in the presence of pRT-T1 demonstrated significantly greater CAT activity than those electroporated with buffer alone. Elevated CAT activity was not detected in the sample electroporated with pAP-S13.

0, 1 and 3 show positions of chloramphenicol and its monoacetylated forms.

Figure 4.7


A.
3
1
0
3
1
0
RTCAT100
UPS92E
APcat1
92J-500
RTUPS600
92J-500
SCOX920
UPS92E
B.
3
1
0
AP-S13
LTE
RT-T1

4.5 The COXI-CAT Fusion Protein Retains CAT Activity.

The 5' end of the CAT coding sequence in pUPS92J and in pSCOX920 is fused in frame to a sequence encoding five amino acid residues from the N-terminus of COXI and four residues from a linker sequence (Figure 3.9B). Although it retains its own initiation codon, there is a possibility that, if translated in mitochondria, the vast majority of initiations would use the *coxI* initiation site. If so, most CAT molecules would carry an N-terminal extension, and were this to inactivate the protein, little or no resistance would ensue. An inactive protein would provide an alternative explanation for the results obtained in the previous two sections. A search of the literature on CAT enzymology found no evidence concerning the tolerance of the *Proteus* CAT to such extensions. Goldfarb *et al.* (1981) provided circumstantial evidence that such extensions are tolerated by Tn*9* encoded CAT in *Bacillus subtilis*. Subsequently, French *et al.* (1986), Boutry *et al.* (1987), and Takamatsu *et al.* (1987) have shown that activity is retained by several derivatives of ~~the~~ this CAT variant which almost certainly carry N-terminal extensions. It was therefore necessary to prove that the *Proteus* CAT would be similarly tolerant of the nine amino acids from *coxI* and pUC9 at its N-terminus.

Choice of a Suitable Expression System.

If active CAT is synthesised from the fused coding sequence in any particular expression assay, its synthesis cannot simply be interpreted as evidence that the fusion protein retains activity because the active molecules may have been translated from the initiation codon of either *coxI* or *cat*. As the results obtained with pUPS919 and pSCOX911 in section 4.3 clearly demonstrate, initiation from the internal ATG codon is particularly likely in bacterial cells where selection of the initiation codon is complex (Kozak 1983). In contrast, translation of eukaryotic mRNA is generally thought to initiate at the first ATG codon that the ribosomes encounter as they move from the 5' to the 3' end of the molecule (Kozak 1983, 1986a). This would clearly simplify interpretation of results obtained with the COXI-CAT fusion. However results obtained with animal cells and discussed by Kozak (1986b) show that if the first ATG codon is not part of a consensus sequence that promotes efficient initiation, some ribosomes may pass it and initiate translation at a second codon that is in such a consensus.

Similar observations have been made with plant cells. The coding region of *nptII* in the initial kanamycin resistance marker successfully used for plant cell transformation (Herrera-Estrella *et al.* 1983b) was preceded by an second initiation codon out-of-frame with the coding region. The sequences surrounding neither one of these codons showed significant homology to either the consensus sequence for initiation derived from eukaryotic mRNAs by Kozak (1986b), or one

derived specifically for plants (Lütcke *et al.* 1987, Joshi *et al.* 1987). When this out of frame codon was removed (Bevan 1984, Rogers *et al.* 1985), expression of *nptII* increased, and the highest kanamycin concentration at which transformants remained resistant increased from 150 μg/ml to 750 μg/ml indicating that although plant cytosolic ribosomes can initiate translation at a second ATG codon, they probably do so less frequently than at the first.

Because of the implications of these observations for expression of the COX-CAT fusion, the following experiment was performed using transient expression in tobacco protoplasts.

The initial experiment was to discover whether or not the fused open reading frame could direct any CAT activity irrespective of the initiation codon used. To do this a 1 kb *Hae*III fragment containing the fusion was removed from pUPS92J and inserted into the expression vector pRT101 (Töpfer *et al.* 1987, Figure 4.8). This fragment contains the whole fused reading frame plus about 50 bp of the *coxI* leader upstream, and 200 bp of pUC9 downstream (Figures 4.8 and 3.9). Plasmid pRT101 was digested at its unique *Bam*HI site between the Cauliflower Mosaic Virus (CaMV) 35S promoter and polyadenylation site; the resulting termini were made double stranded with T_4 DNA polymerase, and were then ligated to the *Hae*III fragment. This fragment has cytosine residues at it's 5' ends and so regenerates the *Bam*HI sites upon insertion, allowing the reactions to be checked by restriction endonuclease digestion. *E. coli* transformants obtained with the ligation products were screened for resistance to 4 and 6 μg/ml chloramphenicol, and plasmid DNA isolated from the resistant clones was digested initially with *Bam*HI, and additionally with *Eco*RI, *Hin*dIII, and *Eco*RI with *Hin*dIII as shown in Figure 4.9A. A plasmid with *cat* in the desired orientation for expression from the CaMV 35S promoter was identified and called pRTUPS600 (Figure 4.8 and 4.9A). As a control, *cat* from pCmP92 (Figure 3.9) which lacks the N-terminal *coxI* sequence was removed with *Bam*HI and ligated directly into the *Bam*HI site of pRT101 (Figure 4.8). Recombinants were screened on chloramphenicol as above, and one plasmid with *cat* in the desired orientation was isolated, mapped by restriction endonuclease digestion (Figure 4.9A) and designated pRTCAT100. A second clone designated pRTCAT200 with *cat* in the reverse orientation was similarly recovered and was used as a negative control.

These plasmids were used for transient expression in tobacco mesophyll protoplasts, and compared with pCAP212 that expresses the Tn*9* CAT gene from the 1' promoter of the T-DNA (Velten and Schell 1985). The results are shown in Figure 4.11. Plasmid pRTUPS600 containing the fused coding sequence clearly expresses *cat* at levels comparable with the wild type CAT gene of pRTCAT100, and the Tn*9* gene of pCAP212. The next step was to determine whether the activity measured was derived from hybrid molecules translated from the

Figure 4.8

Construction of Plasmids pRTCAT100 and pRTUPS600 that are Designed to Allow Expression of the *P. mirabilis* CAT Gene and its Fusion to *coxI* from Maize in Tobacco Cells.

pRT101. This is a derivative of the high copy number plasmids pUC18 and pUC19 (Yanisch-Perron *et al.* 1985) constructed by Töpfer *et al.* (1987). It contains the 35S promoter (box with white triangles) and a polyadenylation signal (shaded box) derived from the Cauliflower Mosaic Virus. Between these sequences is a polylinker containing unique recognition sites for several restriction endonucleases that allow the insertion of foreign sequences. The whole expression cassette is flanked by recognition sites for *Pst*I (P), *Sph*I (Sp) and *Hin*dIII (H).

pRTCAT100. This plasmid was constructed by inserting the 760 bp fragment isolated from a *Bam*HI (B) digest of pCmP92 into the *Bam*HI site in the pRT101 polylinker. This fragment contains the coding sequence of the *P. mirabilis* CAT gene (Stippled box, arrow indicates the direction of transcription), plus 10 bp upstream and about 90 bp downstream. The structure of pRTCAT100 was confirmed by restriction endonuclease digestion (Figure 4.9A).

pRTUPS600. This plasmid was constructed by inserting the 1050 bp *Hae*III (He) fragment of pUPS92J (drawn as in Figure 3.9) into the *Bam*HI site of pRT101. The *Hae*III fragment contains the entire chimaeric COXI/CAT open reading frame of pUPS92J, plus 50 bp upstream and 300 bp downstream. The DNA fragments generated by *Hae*III and *Bam*HI are not complementary, so were treated with T4 DNA polymerase prior to ligation. The *Bam*HI sites of pRT101 were regenerated upon insertion of the *Hae*III fragment allowing the polymerase and ligation reactions to be checked. The structure of pRTUPS600 was confirmed by restriction endonuclease digestion (Figure 4.9A).

Pv, Xb, Sm, K, S, E, and Xh indicate recognition sites for *Pvu*II, *Xba*I, *Sma*I, *Kpn*I, *Sac*I, *Eco*RI and *Xho*I respectively. *ori*; origin of replication. Ap^r; β-lactamase gene. *LacZ*; alpha complementation region of the *E. coli* β-galactosidase gene.

Figure 4.8

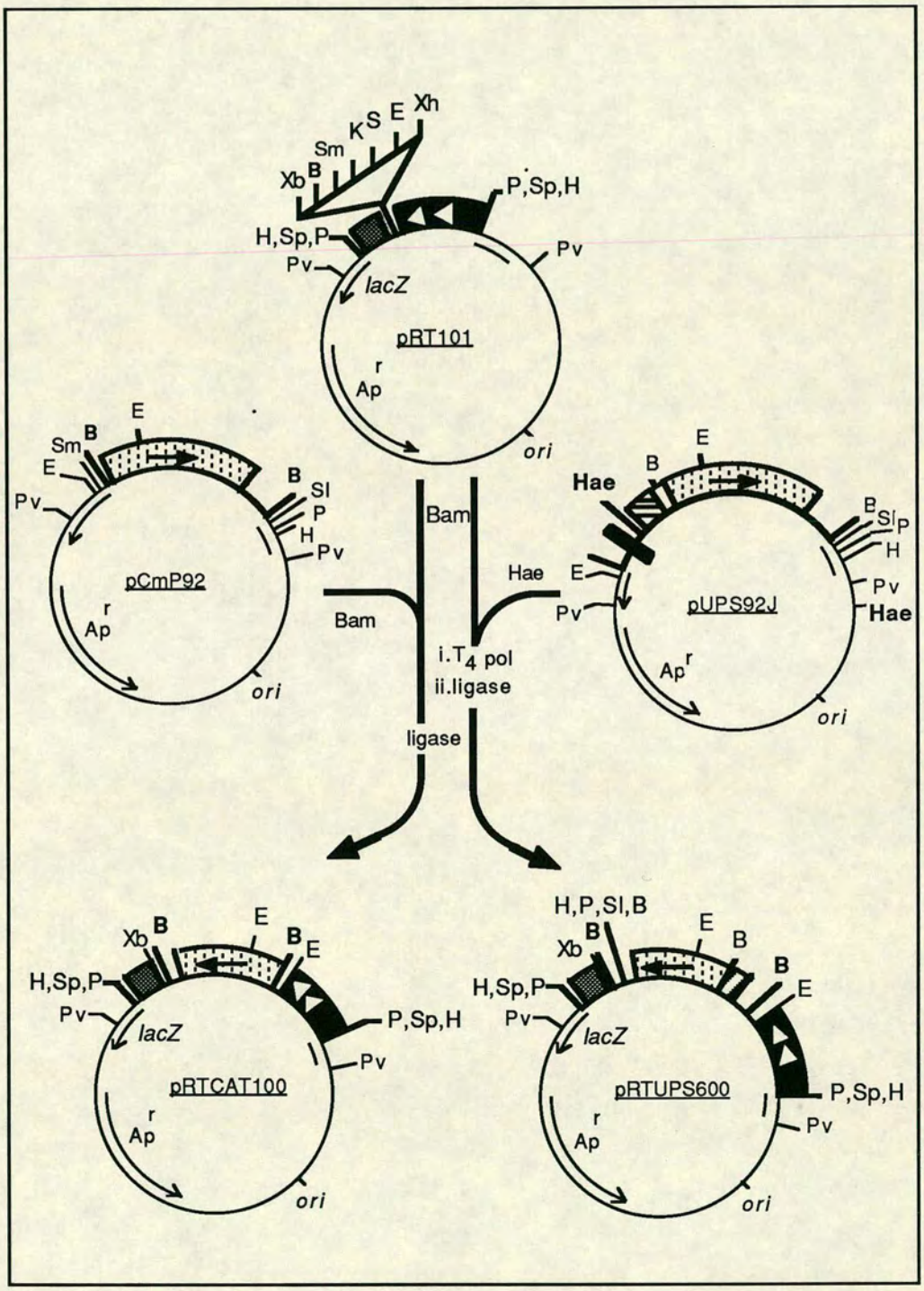

Xh
S
K
Sm
E
Xb
B
P,Sp,H
H,Sp,P
Pv
lacZ
pRT101
r
Ap
ori
Sm
E
B
Sl
P
H
pCmP92
Bam
Hae
i.T4 pol
ii.ligase
ligase
Hae
pUPS92J
H,P,Sl,B
pRTCAT100
pRTUPS600

Figure 4.9 A

Restriction Endonuclease Digestion Analysis of pRTCAT100 and pRTUPS600.

Plasmid DNA was digested with restriction endonucleases, and the digestion products were separated by electrophoresis through an agarose gel. Nucleic acid was stained with ethidium bromide and the gels were photographed under U.V. illumination, all as described in Materials and Methods.

A. This shows the products of digestion of plasmid pRTUPS600 with either *Eco*RI (E), *Hin*dIII (H) or both these enzymes. The approximate sizes of the marker bands (*Hin*dIII and *Hae*III digestion products of bacteriophages λ and ϕX174 respectively) are given to the right. The origin and approximate size in base pairs of the fragments that are expected from each digest are shown in part **D**.

B. This shows the products of digestion of plasmid pRTCAT100 with either *Eco*RI, *Pst*I (P) or *Bam*HI (B), and the products of digestion of pCAP212 with *Bam*HI, *Pst*I or *Eco*RI with *Pst*I. Plasmid pCAP212, constructed by Velten and Schell (1985) was used on several occasions in this work for transformation of plants cells to chloramphenicol or kanamycin resistance. The digestion products observed on this gel were consistent with the structure published by Velten and Schell (1985). The DNA size markers on this gel were the same as those in part **A** and are shown to the right. The origin and approximate size in base pairs of the fragments that are expected from each digest of pRTCAT100 are shown in part **C**.

C. Schematic diagram of the expression cassette of pRTCAT100 drawn similarly to Figure 4.8, and showing the 35S promoter (35S), 760 bp insert (Cat) containing the CAT gene of *P. mirabilis* and the polyadenylation signal (pA). The *Eco*RI digest indicated the orientation of the insert in pRT101. In all digests, an additional vector band of between 2600 bp and 4000 bp is expected.

D. Schematic diagram of the expression cassette in pRTUPS600 drawn similarly to part **C**. The additional sequence derived from the maize COXI encoding region is shown by the hatched box. The *Eco*RI and the *Hin*dIII digests indicated the orientation of the insert in pRT101. The *Bam*HI sites regenerated upon insertion of the 1050 bp *Hae*III fragment are in bold type. In all digests, an additional vector band of between 2600 bp and 4000 bp is expected. In comparison with pRTCAT100, pRTUPS600 contains an additional 200 bp from pUC9 between the 3' end of the CAT encoding sequence and the polyadenylation signal. This was removed by digestion of pRTUPS600 with *Xba*I (Xb) and *Sal*I (Sl), treatment with Klenow fragment of DNA polymerase I, and religation of the vector to produce pRTΔ601 as indicated.

Figure 4.9 A

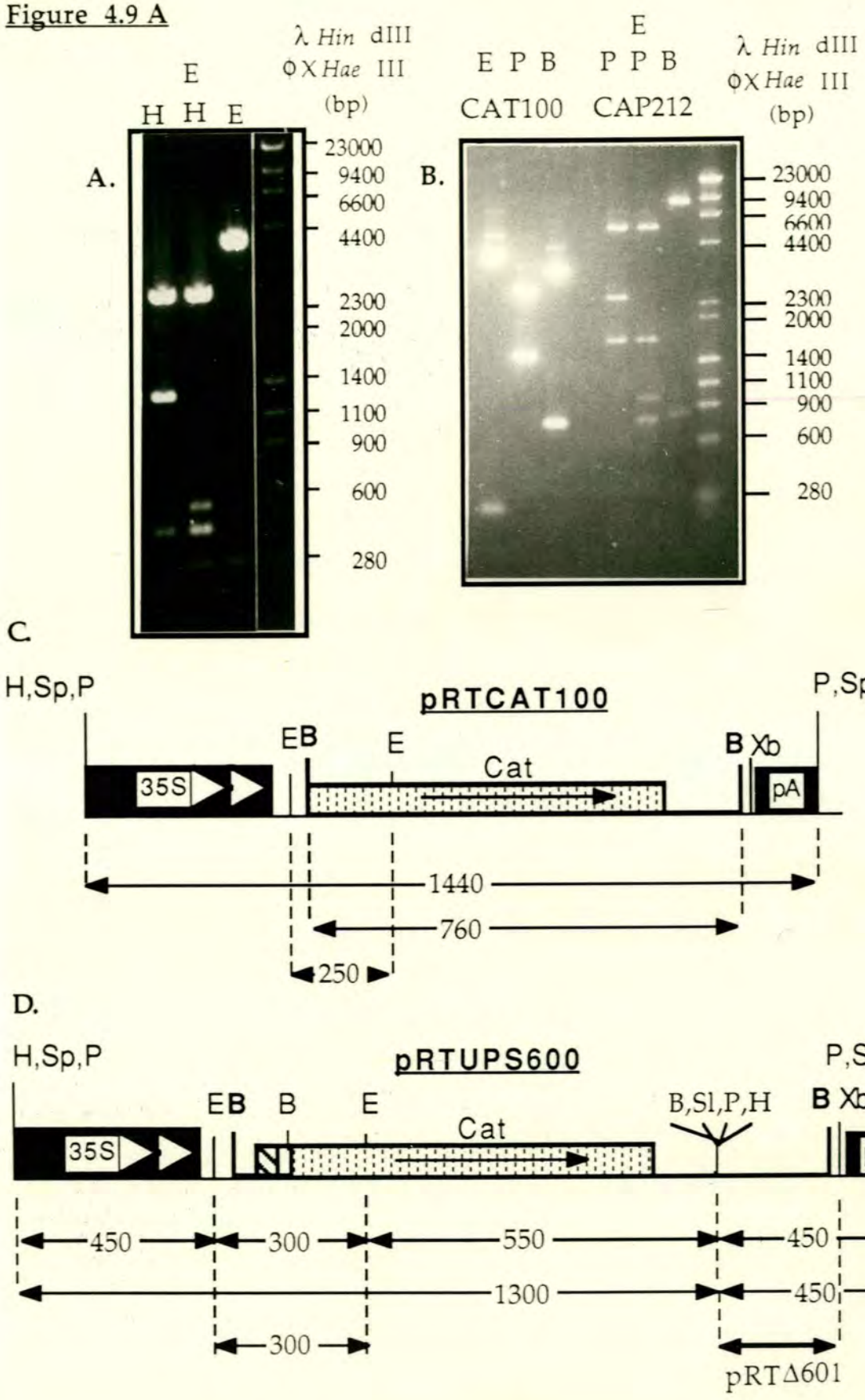

A.
H H E
E
λ Hin dIII
φX Hae III
(bp)
23000
9400
6600
4400
2300
2000
1400
1100
900
600
280
B.
E P B
CAT100
E
P P B
CAP212
λ Hin dIII
φX Hae III
(bp)
23000
9400
6600
4400
2300
2000
1400
1100
900
600
280
C.
H,Sp,P
pRTCAT100
P,Sp,H
EB
E
Cat
B Xb
35S
pA
1440
760
250
D.
H,Sp,P
pRTUPS600
P,Sp,H
EB
B
E
Cat
B,Sl,P,H
B Xb
35S
pA
450
300
550
450
1300
450
300
pRTΔ601

Figure 4.9 B

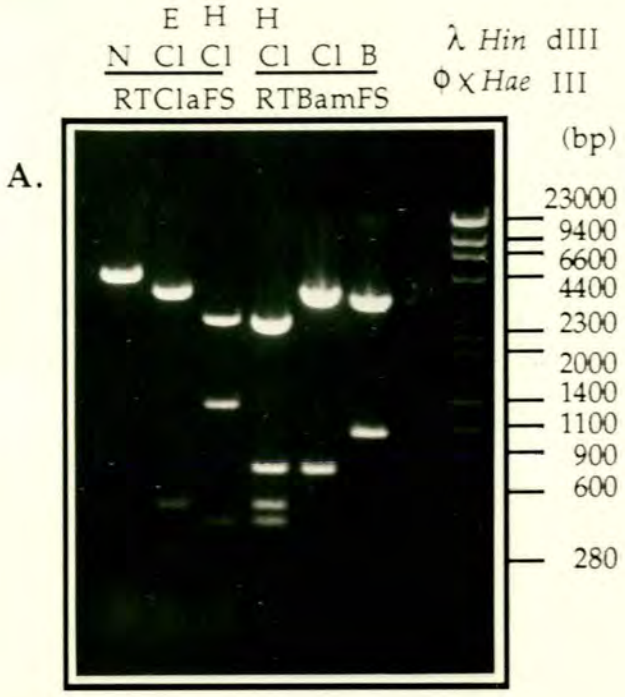
Restriction Endonuclease Digestion Analysis of pRTBamFS and pRTClaFS.

A. This shows the restriction endonuclease digestion products of plasmids pRTBamFS and pRTClaFS separated by electrophoresis through an agarose gel, stained with ethidium bromide and photographed under U.V. illumination. Plasmid DNA was digested with either *Nru*I (N), *Eco*RI (E), *Cla*I (Cl), *Hin*dIII (H) or *Bam*HI (B), or with combinations of these endonucleases as indicated at the top of each lane. The DNA size markers are the same as those used for Figure 4.9A, and their approximate sizes are shown to the right of the figure. The origin and approximate size of the fragments expected in each digest are shown in parts **B** and **C**.

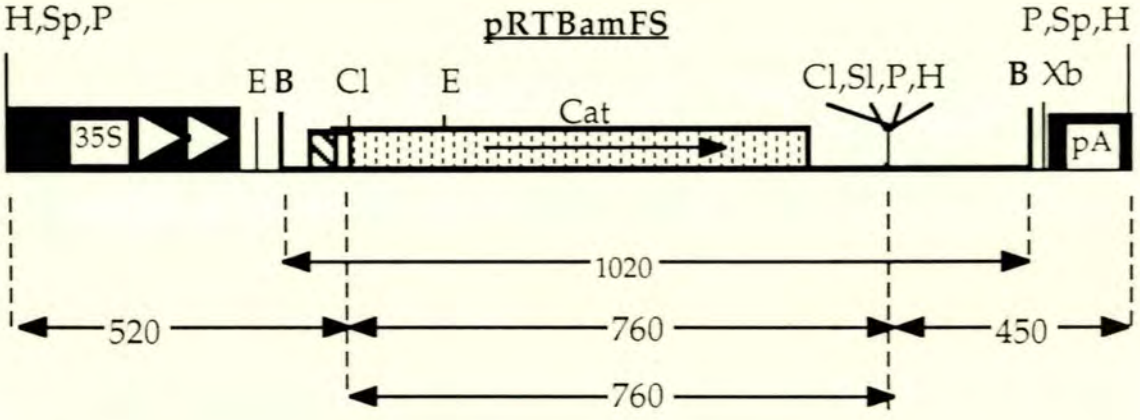
B. is a schematic diagram of pRTBamFS drawn similarly to pRTUPS600 in Figure 4.9A part D. The only difference expected between pRTBamFS and pRTUPS600 is that the two *Bam*HI sites immediately flanking the CAT gene in the latter plasmid are *Cla*I sites in pRTBamFS. Consequently, digestion of pRTBamFS with *Cla* I produced a fragment of approximately 760 bp. Digestion with *Bam*HI produced a larger fragment of 1050 bp due to the regenerated *Bam*HI recognition sites located at the ends of the inserted *Hae*III fragment. The *Hin*dIII and *Cla*I double digest revealed the orientation of the insert (in the reverse orientation, fragments of approximately 760 bp, 700 bp and 300 bp were predicted).

C. is a schematic diagram of pRTClaFS drawn similarly to pRTBamFS in part **B**. This plasmid was derived from pRTBamFS by converting the *Cla*I site at the 5' end of the CAT gene coding sequence into a unique *Nru*I site. Digestion of pRTClaFS with *Nru*I resulted in linearisation of pRTClaFS, but a fragment of approximately 760 bp was not seen indicating that a recognition site had been introduced at only a single position. The 1310 bp fragment produced by digestion with a combination of *Hin*dIII and *Cla*I is not cleaved further by *Cla*I showing that the recognition site for this enzyme at the 5' end of the CAT gene coding sequence is no longer present. The production of an approximately 320 bp fragment upon digestion with a combination of *Eco*RI and *Cla*I confirms the absence of the 5' *Cla*I site, and the 550 bp fragment confirms the presence of the 3' *Cla*I recognition site.

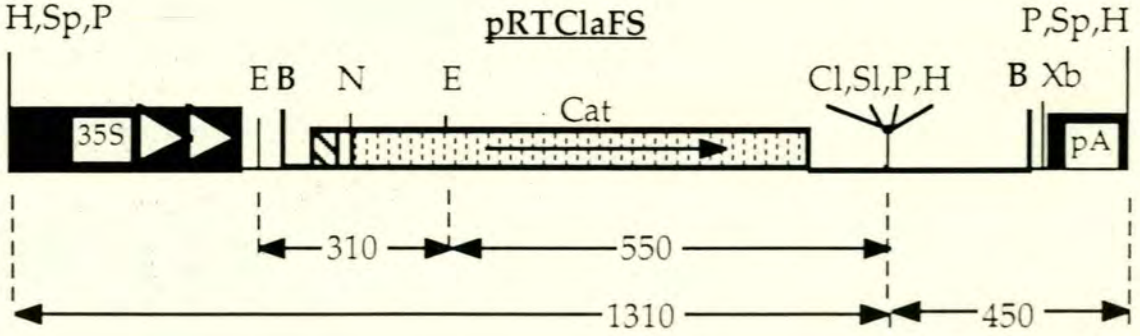
Figure 4.9 B



B.



C.



initiation codon of *coxI* or from wild type CAT molecules translated from the initiation codon of *cat* .

Given that both the wild type and fused gene products are likely to be translated in plant cells, it would be difficult to directly demonstrate activity from the hybrid protein. Instead it was decided to observe the loss of activity that ensues when a frame-shift is introduced between the two potential initiation codons allowing the CAT open reading frame to be translated from only the second initiation codon. As translation from the initiation codon of *coxI* would no longer produce active molecules, the difference between the CAT activity expressed by the in-frame and out-of-frame constructs can be attributed to the fusion protein.

To insert the frame shift mutation, pUPS92J which contains the COXI-CAT fusion was digested at its *Bam*HI sites (Figure 3.14B), the termini were made blunt ended with T_4 DNA polymerase, and the resulting molecules were religated. After transformation of a *dam*$^-$ strain of *E. coli* provided by Dr. C. Koncz, Max Planck Institut Köln, clones carrying plasmids yielding an approximately 760 bp *Cla*I fragment were isolated. This enzyme recognises the sequence 5' ATCGAT 3', which occurs in the sequence 5' GGATCGATCC 3' that results from religation of the blunt ended *Bam*HI fragments. A clone carrying *cat* reinserted in the same orientation as in pUPS92J was identified by *Eco*RI digestion and called p92JBamFS (Figure 3.14B). The *Hae*III fragment containing the CAT gene of this plasmid was transferred into pRT101 to produce plasmid pRTBamFS by a procedure identical to that used above for construction of pRTUPS600 (Figures 4.8 and 4.9B). This plasmid is identical to pRTUPS600 except that it has 4 bp inserted at each end of *cat* which place the gene in a different reading frame to that of the *coxI* initiation codon, and introduce a termination codon between them (Figure 4.10A).

The only two *Cla*I sites in pRTBamFS are those flanking *cat*. If these were digested, then filled in with DNA polymerase and religated, two more base pairs would be inserted between the two initiation codons which would return them both to the same reading frame (Figure 4.10A). This was attempted, and plasmid DNA from transformants screened for the presence of *Nru*I sites that should be generated in place of the *Cla*I sites. Introduction of these *Nru*I sites was only partially successful, perhaps due to incomplete digestion or polymerisation at the ends. However, one plasmid called pRTClaFS was recovered which had an *Nru*I site at the 5' end of *cat* but retained the *Cla*I site at the 3' end (Figure 4.9B). The sequence between the *coxI* and *cat* initiation codons in this construct is shown in Figure 4.10A.

Transient expression directed by these plasmids was assayed in tobacco protoplasts, and the results are shown in figure 4.11 part C. Firstly it can be seen that pRTUPS600 was expressed as expected in the assay. Secondly, pRTBamFS consistently induces CAT activity above background, confirming previous

observations of translation from an internal initiation codon. The activity expressed from this plasmid is much reduced relative to pRTUPS600 and pRTClaFS, and the additional activity expressed by the latter two plasmids is likely to be due to translation of active molecules from the *coxI* initiation codon. CAT activity appears to be completely restored by the second frame shift in pRTClaFS, although some variation was observed in the relative CAT activities produced by these plasmids in separate experiments (see also section 4.4.2.1 and Figure 4.6B). Relative to pRTUPS600, pRTBamFS contains sequence alterations around the 5' and 3' end of *cat*; however as pRTClaFS was generated by further modifying pRTBamFS at only the 5' end of *cat*, the reduction in the CAT activity expressed by the latter plasmid can be more confidently attributed to the mutation between the two initiation codons.

Similar experiments have been performed by French *et al.* (1986) and Takamatsu *et al.* (1987) with similar results. They analysed transient expression from constructs that potentially synthesise CAT fused by its N-terminus to the first few residues of two different viral coat proteins (Figure 4.10, constructs B1 and C1). The CAT gene in these constructs retained its initiation codon as in the plasmids described above. Frame shift mutations that introduced a termination codon between the two potential initiation codons (constructs B2 and C2) resulted in reduction, but not abolition, of activity as with pRTBamFS. It was concluded that the fusion protein was being synthesised and contributing to the total CAT activity. French *et al.* (1986) estimated the activity synthesised from the out-of-frame construct to be 65 % of that of the in-frame one. The relative CAT activities induced by pRTUPS600, pRTBamFS and pRTClaFS were esimated by scanning the autoradiographs using a Quick Scan R&D densitometer (Helena Laboratories). The relative activities of each plasmid in each of the three duplicate experiments, and the means for these values were determined. The means were 90%, 25% and 100% for pRTUPS600, pRTBamFS and pRTClaFS respectively, which are qualitatively similar to the results of French *et al.* (1986). The relative CAT activities induced in protoplasts by these three plasmids in electroporation experiments (100%, 50% and 75% respectively, Figure 4.6B iii) are quantitatively more similar to those of French *et al.* (1986). These results do not allow a confident quantitative estimation of the relative CAT activities induced by each plasmid, though it is clear that pRTBamFS consistently induces greater CAT activity than the negative controls, but less than either of the in-frame constructs. Quantitative similarity between the results obtained with the plasmids used in this thesis and those used by French *et al.* (1986) is not to be expected as the relative expression levels will depend upon the efficiency with which translation initiates at the upstream and downstream ATG codons, and this will probably differ for the two sets of constructs.

Interestingly, French *et al.* (1986) also determined the activity synthesised

Figure 4.10

Frame Shift Mutations Inserted Between the Authentic Initiation Codon of *cat* and an alternative Upstream Initiation Codon.

The sequences around the junction of various fusions between *cat* and either *coxI* of maize (A) or viral coat protein genes (B and C) are shown. The nucleotide sequence of the coding strand is shown. Initiation codons for either the fusion (marked 1.) or wild type product (marked 2.), and the introduced termination codons are in heavy type. The predicted products of translation from each initiation codon are shown in single letter code below each sequence. Termination is indicated by an asterisk.

A. The relevant sequences of the plasmids described in the text are shown; translation from the first initiation codon can continue through *cat* in only pRTUPS600 and pRTClaFS. The *Bam*HI, *Cla*I and *Nru*I recognition sites that were used to generate and confirm the frame shifts are underlined in the pRTUPS600, pRTBamFS and pRTClaFS sequences respectively.

B. The relevant sequences from an analogous pair of constructs described by Takamatsu *et al.* (1987) are shown. The Tn*9* encoded CAT gene was fused to the N-terminal coding region of the Tobacco Mosaic Virus coat protein gene; in construct number 2 as (in pRTBamFS) translation from the upstream initiation codon is out of frame with *cat* and terminates before its initiation codon.

C. The relevant sequences from an analogous set of constructs described by French *et al.* (1986) is shown. The Tn*9* encoded CAT gene was fused to the N-terminal coding region of Brome Mosaic Virus coat protein gene. The first two constructs are analogous to the first two in parts A and B. In the third translation from upstream continues past the initiation codon of *cat* but out of frame. The relative CAT activities generated by each construct in a transient expression system are shown on the left.

Figure 4.10

Frame Shift Mutations Inserted Between the Authentic Initiation Codon of *cat* and an Alternative Upstream Initiation Codon.

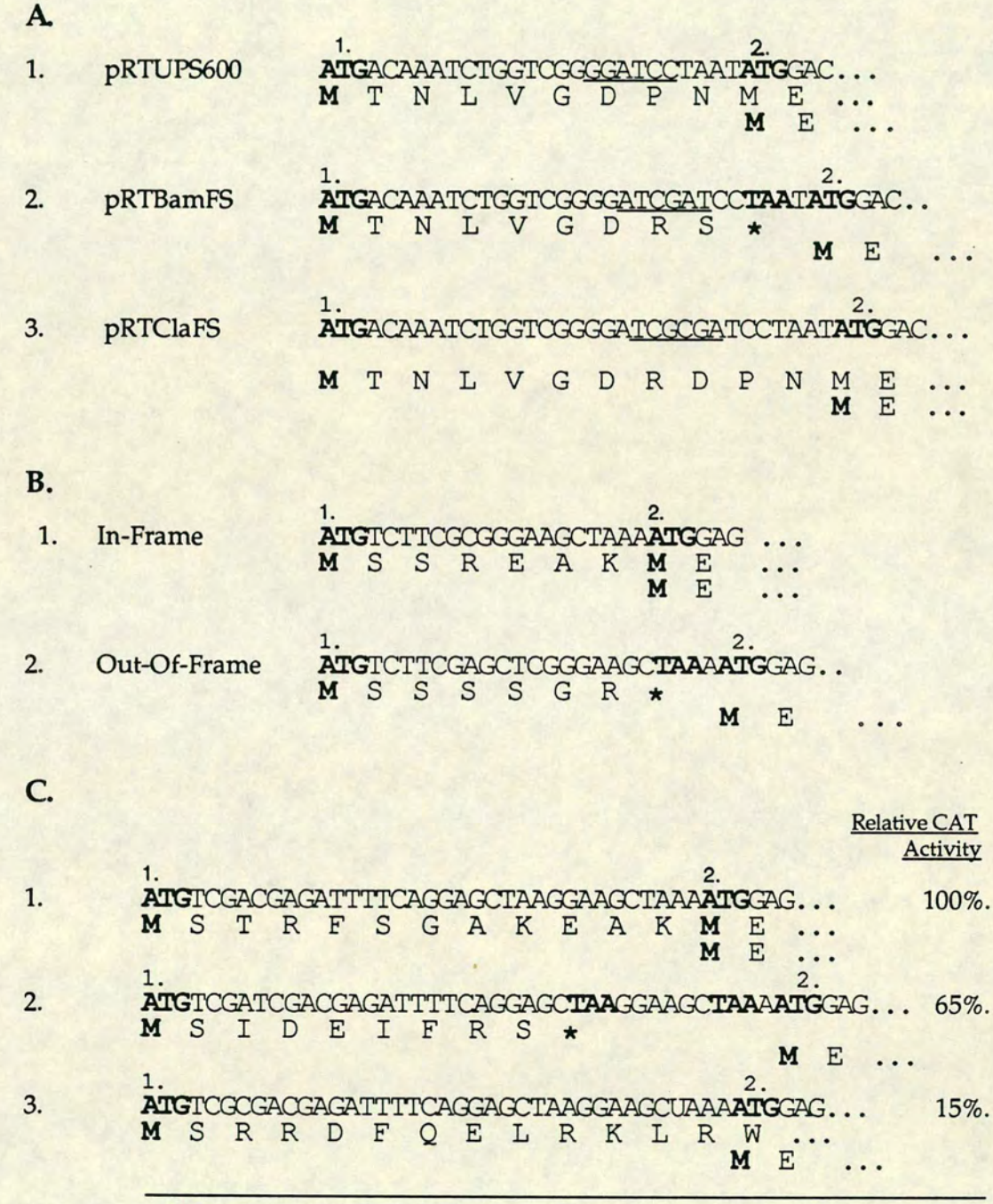


Figure 4.11

Transient Expression in Tobacco Protoplasts Using Plasmids pRTCAT100, pRTUPS600, and Their Derivatives.

A. The four lanes at the right show the CAT activity that resulted when the PEG-calcium nitrate transformation procedure was used to transform samples of $4x10^5$ tobacco leaf mesophyll protoplasts with 40 μg of supercoiled DNA of plasmids pRTUPS600 (600), pCAP212 (212), pRTCAT200 (200) or with 40 μl of buffer lacking DNA (LTE). Protoplast survival was estimated to be about 25%. Assays of CAT activity were performed three days after transformation. The two lanes at the left are derived from a repeat of the transient expression assay with plasmids pRTUPS600 and pCAP212. Use of both of these latter plasmids resulted in significant CAT activity accumulating in the protoplasts, though none was detected when the negative controls LTE or pRTCAT200 were used.

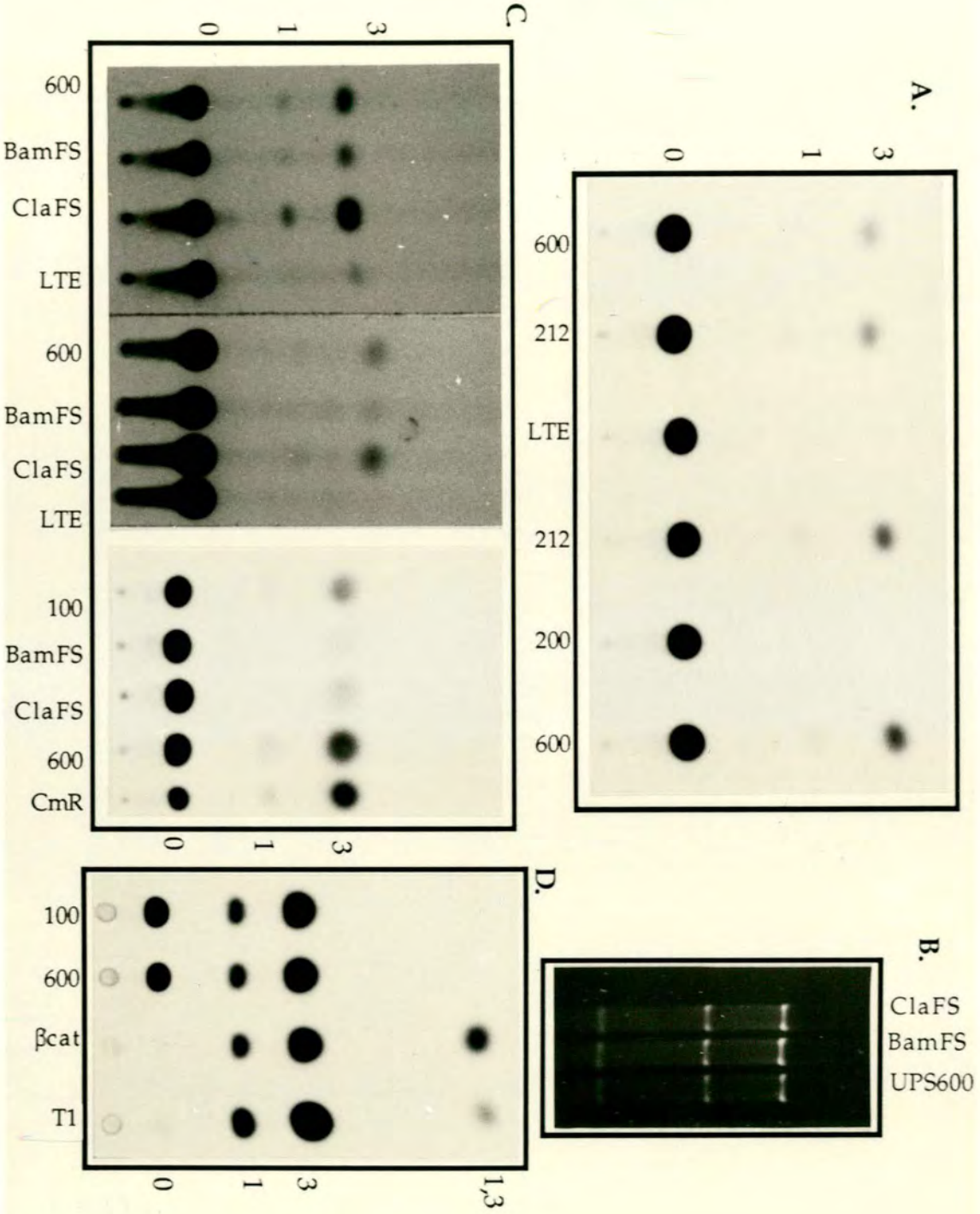
B shows a *Hin* dIII digest of an aliquot of the DNA preparations used in the transient expression assays shown in **C**. DNA was separated by agarose gel electrophoresis, stained with ethidium bromide and photographed under U.V. illumination. ClaFS, BamFS and UPS600; pRTClaFS, pRTBamFS and pRTUPS600.

C. The four lanes at the left show transient expression of the CAT gene in approximately $5x10^5$ tobacco leaf mesophyll protoplasts transformed using the PEG-calcium nitrate procedure with 20 μg of DNA from the preparation shown in **B** or with 40 μl of buffer (LTE). The next four lanes show a repeat of this experiment using the same DNA preparations to transform approximately ten thousand protoplasts. The last five lanes show transient expression in approximately $1x10^6$ protoplasts using 40 μg of a different preparation of the same three plasmids and in addition, pRTCAT100 (100). CmR shows CAT activity in callus of a chloramphenicol resistant transformant recovered using pCAP212.

D. Approximately $1x10^6$ protoplasts were transformed with 40 μg of supercoiled plasmid pRTCAT100, pRTUPS600, pRTpreβcat (βcat) or pRT-TI (T1). The latter two plasmids are pRT101 derivatives containing the Type I CAT gene (Figure 6.6A and B). The CAT activity in each sample was assayed and is shown for comparison.

0, 1, 3, and 1,3 indicate the positions of chloramphenicol, its two monoacetylated forms and its diacetylated form respectively.

Figure 4.11


A.
0
1
3
600
212
LTE
212
200
600
B.
ClaFS
BamFS
UPS600
C.
0
1
3
600
BamFS
ClaFS
LTE
600
BamFS
ClaFS
LTE
100
BamFS
ClaFS
600
CmR
0
1
3
D.
100
600
βcat
T1
0
1
3
1,3

from a third construct, C3, which had a different frame shift that did not introduce a termination codon before the initiation codon of *cat* . In this construct translation from the upstream initiation codon continues past that of *cat* but out of frame. Interestingly this construct produced only 15 % of the activity of the in-frame one. The higher activity expressed by construct C2 was presumably due to translation from the first initiation codon being terminated at the introduced termination codon, but then reinitiating at the begining of *cat* . This was the first report of such reinitiation, though it has been proposed for some viral reading frames (B. Gronenborn, personal communication). Its relevance to the experiment above is that much of the activity expressed by pRTBamFS may also be due to translation from the initiation codon of *coxI* , which then terminates and is reinitiated at the begining of *cat* . Such reinitiation reduces the apparent effect of the frame shift and over-estimates the contribution that wildtype CAT makes to the activity expressed by pRTUPS600 in which translation does not terminate before *cat*. Therefore the CAT fusion protein almost certainly retains enzyme activity; in fact, on the basis of the results of French *et al.* (1986), if the fusion were inactive pRTBamFS should express more activity than either pRTUPS600 or pRTClaFS.

Alternative interpretations of these results are that either all translation products, or all active molecules originate from the second initiation codon, and that the mutations introduced simply affect the relative efficiency of translation of the mRNA from this site. This is unlikely because the frame shift mutations lie outside the regions predicted by Kozak (1986a and b), Lütke *et al.* (1987) and Joshi (1987) to influence initiation frequencies at either codon. Furthermore, translation of active molecules from the first initiation codon is the simplest explanation for restoration of activity in pRTClaFS. This alternative interpretation could be investigated by determining the effect of the same mutations on CAT expression from pRTCAT100 which lacks the *coxI* sequence, or by using CAT specific antisera to determine the relative amounts of wild-type and hybrid protein that are synthesised from each of these plasmids.

4.6 Conclusion.

In this chapter the characterisation of the mitochondrial transformation vectors described in Chapters 3 and 5 has been described. The sequence of the fused open reading frame derived from *coxI* and *cat* in pUPS92J and pSCOX920 has been confirmed. Evidence has been presented from transient expression in protoplasts that this fusion protein retains CAT activity, and that it functions in the cytosol of plant cells. After stable transformation of the nucleus, or in

transient expression assays, no evidence has been found, for expression of *cat* in the mitochondrial transformation vectors by the nuclear and cytosolic gene expression system. These results imply that the mitochondrial gene expression signals may provide the desired specificity of CAT gene expression. There appears to be a relatively low level of expression of *cat* from the mitochondrial transformation vectors in bacterial cells, though it remains to be seen whether this will lead to cross protection and recovery of nontransformed escapes when *A. tumefaciens* is used to transform plant cells.

CHAPTER 5.

MAINTENANCE OF THE TRANSFERRED DNA MOLECULES IN MITOCHONDRIA OF TRANSFORMED PLANTS.

5.1. Introduction.

The work described so far has been confined to the construction of mitochondrial transformation vectors that carry a potentially selectable chimaeric mitochondrial gene. In succeeding chapters the delivery and selection of these genes will be discussed, but for cells to survive the selection process, the transferred DNA must be maintained for several cell generations, and attempts to ensure that this occurs are described here. Two approaches have been considered; independent replication of transferred DNA, and its integration into the mitochondrial genome of the transformed plant.

5.2 Maintenance of the transferred DNA Sequences as Part of the Mitochondrial Genome.

Replication and inheritance of the transferred DNA molecules with the main genomic molecules, requires some form of recombination event to link them. Evidence of such recombination events in plant mitochondria has been frequently reported. Most events that have been analysed have been attributed to reciprocal recombinations between homologous sequences, and the evidence from a variety of sources is discussed below.

Recombination Between DNA Sequences in Plant Mitochondria.

As described in Chapter 1, the mitochondrial genomes of several species carry sequence duplications of up to 14 kb. Recombination between these repeated

sequences appears to have occurred as the unique sequences flanking each of the repeats have been found in all the pairwise combinations that are predicted to result from recombination (Palmer and Shields 1984, Isaac *et al.* 1985a, Lonsdale *et al.* 1984, Schuster and Brennicke 1986, Falconet *et al.* 1984, Quetier *et al.* 1985).

In maize mitochondrial DNA, a recombination event appears to have been responsible for transposing a 186 bp sequence to different sites within N- and S-Type genomes (Isaac *et al.* 1985b). This sequence has homology to the terminal inverted repeats of the linear plasmids S1 and S2, and in S-type cytoplasms it appears to recombine with each end of the two plasmids to generate an abundance of recombinant forms (Schardl *et al.* 1984, Schardl *et al.* 1985, Leaver *et al.* 1985, and Braun *et al.* 1986). Evidence has also been found of recombination between the homologous portions of S1 and S2 (Paillard *et al.* 1985), and within minicircles of sugarbeet (Thomas 1986). Furthermore, Smith and Pring (1987) working with maize, and Thomas (1986) with sugarbeet, have further shown that such minicircular DNAs can exist as multimers of up to 6 copies that presumably arise by recombination, and Dale *et al.* (1983) have made similar observations with a 10.1 kb subgenomic circle from tobacco mtDNA.

Finally, sequences present in mitochondrial DNA that have homology to plastid and nuclear DNA are supposed to have arisen by transfer from these genomes, in which case some form of recombination event must have been involved (Stern and Lonsdale 1982, Lonsdale *et al.* 1983, Bedinger 1987, Schuster and Brennicke 1987a,b,c). It is not clear what sort of events these were, but there is no suggestion in these cases that simple homologous recombination was responsible, and Schuster and Brennicke (1987a) have proposed that reverse transcription of an RNA intermediate may have been involved.

All of these observations provide persuasive, if circumstantial, evidence that recombination occurs in plant mitochondria, although they provide no estimate of its frequency. In fact, there is no firm data on which to base such an estimate, and the popular supposition that recombination is continually occurring seems to be based on the widespread observation of its presumed products rather than studies of recombination kinetics.

The Frequency of Recombination Events in Plant Cells.

The frequency of recombination would be expected to vary considerably between events involving different sequences, but bearing this in mind, there is evidence from two tissue culture systems that events similar to those discussed above may occur at intervals similar for example to the generation time of a cell.

When callus is initiated from CMS T maize lines, it retains the sensitivity to

methomyl and T-toxin that characterise the parental plant. After a few months, plants can be regenerated that are resistant to these substances and are fertile (Brettel *et al.* 1980, Umbeck and Gengenbach 1983). Rottmann *et al.* (1987) have shown that in the majority of cases this arises through recombination between two 55 bp repeats that are at least 5 kb apart, resulting in the deletion of T-*urf13* which is thought to be responsible for the CMS T phenotype. This occurs in the absence of selection for methomyl or T-toxin resistance, and Brettel *et al.* (1980) found that after 12 months in culture, almost 60% of the plants regenerated from callus were revertants. Umbeck and Gengenbach, and Kuehnle (1987) have made similar observations. Kuehnle (1987) has shown that for periods of up to 16 months the percentage of revertants increases with time in culture before regeneration is initiated, and have excluded preferential regeneration of revertant plants as a source of bias. The revertant phenotype in most plants is stable, although some with intermediate phenotypes segregate in the progeny, and probably have chimaeric mitochondrial genome populations. Reversion from CMS T has never been observed in the field. One interpretation of these results is that over a period of one or two years, recombination has been sufficiently prevalent between the relatively short repeats flanking T-*urf13* to have led to its deletion from most mitochondrial genomes in 60% of the cells in the callus. However, the methods used in these studies do not preclude the possibility that the initial recombination event was rare, but that its products have been amplified in some callus lines either selectively or by random segregation.

The second source of evidence supporting frequent recombination in mitochondria comes from fusion of protoplasts *in vitro*. As mitochondria are normally maternally inherited they are genetically isolated from those of other individuals. By fusion of protoplasts, mitochondria from different individuals, cultivars or species can be brought together in hybrid cells. Belliard *et al.* (1979) analysed hybrids between two parental *N. tabacum* varieties whose mitochondrial DNA restriction endonuclease digest patterns were distinguishable. They showed that the hybrids possessed some restriction fragments that were characteristic of each of the two parental genomes, but that the overall pattern could not be explained by a simple combination of these genomes. Significantly, novel DNA fragments not present in either parental genome were also detected; moreover, different hybrids from the same two parents were found to yield distinguishable mitochondrial DNA restriction endonuclease digestion patterns. The interpretation of these results was that novel DNA fragments were generated in the initial fusion product by recombination between parental molecules, followed during regeneration by segregation of the novel fragments and characteristic parental fragments to produce a variety of different genome structures. These structures may be constrained only by the need to maintain a complete and functional genome. These results have been confirmed for tobacco (for example,

Nagy *et al.* 1981, Galun *et al.* 1982) and extended to include *Brassica* (Vedel *et al.* 1986), carrot (Kothari *et al.* 1986), potato (Kemble *et al.* 1986) and *Petunia* (Boeshore *et al.* 1983, Rothenberg and Hanson 1987a). It has been shown that different regenerants from a single fusion event between *Petunia* protoplasts can differ in their mitochondrial genome structure (Clarke *et al.* 1986).

Rothenberg *et al.* (1985) and Vedel *et al.* (1986) have isolated novel mtDNA fragments as cosmid clones from *Petunia* and *Brassica* hybrids respectively, and shown that they contain sequence that is unique to each parent, but joined by a region of homology. The latter authors showed several sites in the genome to be involved in such recombination events, with some sites apparently preferred over others. Clarke *et al.* (1986) and Nagy *et al.* (1983) found that novel genome structures were not observed in normal sexual hybrids, nor did they appear to result from the tissue culture system used. Furthermore, novel mtDNA fragments could not be detected in hybrids produced by fusion between protoplasts from the same individual, or by interspecific fusions between protoplasts with indistinguishable mtDNA structures. This suggests that for genome rearrangement to occur after hybridisation, a heteroplasmic state must be induced, and that novel intragenomic recombination events do not greatly contribute to the generation of the new genome structures. Also, Vedel *et al.* (1986) analysed several novel mtDNA fragments and showed them all to hybridise to fragments of different size in each parent as would be expected if they were simple recombination products; however no systematic analysis has been undertaken to show that novel fragments are generated solely from genomic regions that differ between the two parents.

The value of these observations is firstly that they increase confidence in there being a degree of flexibility in genome size and organisation that will accommodate new sequences following transformation, but more importantly they provide a time scale for the recombination events. Although Belliard *et al.* (1979) and Izhar *et al.* (1983) found evidence of segregation of mtDNA in the sexual progeny of hybrids, the repeated observations of other authors (cited above) indicates that the mitochondrial DNA structures of the hybrids are generally stable during both vegetative growth and sexual reproduction. This, coupled with the isolation from a single callus of three plants with indistinguishable but novel genome structures (Nagy *et al.* 1981) implies that by the time plant regeneration is initiated, the majority of the novel recombination events have occurred, and their products have segregated to reach a stable equilibrium.

The limitation inherent in these observations is that they do not exclude the possibility that the novel structures may not be generated *de novo* following cell fusion but may already exist at a low level in one or other cell line and simply become amplified. This may apply just as well to the fragments that are characteristic of one cell line and apparently acquired by another. In fact Small *et*

al. (1987) have shown such substoichiometric molecular arrangements to exist in maize cell lines, and have proposed that their amplification is involved in genome evolution (Leaver *et al.* 1988, Small *et al.* 1989). Ozias-Akins *et al.* (1988) have found that apparently new fragments generated in intergeneric somatic hybrids between *Panicum* and *Pennisetum* were present at low copy number in one of the parents. They found one fragment that is present at low copy number in both parents to be the product of recombination across a repeat, and suggest that amplification occurs by preferential recombination across this repeat in the hybrid. They have not however fully explained the recombination event, nor can they exclude other mechanisms for amplification such as preferential replication, or random segregation (see below).

Therefore the generation of novel mtDNA structures can be explained by invoking either *de novo* recombination or amplification of preexisting recombinant forms. Models involving amplification events are complicated and constrained by the observations that 1. new genome structures are detected only when the parental mitochondrial genomes are distinguishable, 2. that new fragments seem to be products of recombination between fragments characteristic of one or both parental genomes, but do not to include sequences characteristic of other genome organisations and 3. they seem not to have undergone recombination or modification events other than those that appear simply to have linked characteristic parental sequences. Any amplification mechanism would require that the mtDNA population of each individual contained representatives of all possible products of recombination between its own mtDNA and that of any other variant in a wide variety of species and genera. It is necessary to propose that these recombined molecules are propagated without undergoing sequence divergence that could be detected by the restriction endonuclease digestion analyses that have been performed. Finally, the amplification mechanism would need to be sufficiently selective to discriminate between those recombination products that contain the sequence organisation of only the two parental genomes, and those that alternatively or additionally contain the sequence organisation of a third genome. *De novo* recombination is the mechanism that most simply explains these results, but it's involvement is not firmly established.

Amplification and Propagation of the Transferred Sequence Following Recombination with the Mitochondrial Genome.

After recombination the transforming DNA will be present most probably at only one site in one copy of the many mitochondrial genomes in the cell. Stable

transformation of tissues derived from this cell requires that one copy of the recombined genome must be inherited by each daughter cell upon division. For this to occur, and perhaps even for a sufficient level of antibiotic resistance to be achieved, the transferred selectable marker gene may have to become amplified, perhaps even to replace the wild type copy of the integration site. How might this be achieved? As described below, there are observations to suggest that such an amplification event may occur, even without selection pressure, but there is very little to indicate the precise mechanisms that would be involved. The difficulty in understanding or predicting such a process arises from the following observations 1. the genome probably exists as several subgenomic molecules 2. each component of the genome may be represented upon several different such molecules 3. the degree of genetic exchange that occurs between the subgenomic molecules is uncertain 4. it is not known how many of these molecules carry origins of replication or how many origins are used at each round of replication 5. that the partitioning mechanism and the number of molecules transferred to each daughter cell are similarly unknown.

Despite this, several empirical observation suggests that amplification and maintenance of the transferred sequences may occur. As previously described, following reversion of CMS T maize callus or fusion of protoplasts from a variety of species, it was possible to recover plants and calli were shown to segregate for mitochondrial characters. This alone implies that one copy of each mtDNA molecule within a cell is not passed to each daughter cell at division, but rather suggests that partitioning and perhaps replication of the mtDNA population occurs at random. A further example is provided by the work of Schardl *et al.* (1985) who studied five fertile revertants arising from CMS S cytoplasms. They found that the genome of each was the product of a novel set of recombination events each of which had presumably occurred once and then become established during subsequent cell divisions.

The Non Chromosomal Stripe (NCS) phenotype of maize, described in section 1.1 has been shown to correlate with an alteration of mtDNA. This altered form of the genome, normally exists in a mixed population with the wild type arrangement, and these two are continually segregating at mitosis and meiosis to produce respectively necrotic sectors or progeny afflicted to varying degrees. In extreme cases it is either seedling lethal, or the progeny appear normal and permanently cured (Newton and Coe 1986).

It has already been mentioned that different somatic hybrids from the same two parents have mtDNA structures that usually differ from each other and from the parental genomes, suggesting that segregation from a heterogeneous population can occur rapidly. Furthermore, the the novel genome structures are stably maintained in the somatic hybrids and the various maize lines discussed above (Schardl *et al.* 1985, Newton and Coe *et al.* 1986, Small *et al.* 1987),

indicating that segregation can lead to either a static genome structure, or a population of molecules in stable equilibrium.

It is noteworthy that these segregation events seem to occur in the absence of any discernible selection pressure other than the obvious requirement for retention of mitochondrial functional. How the application after transformation of positive or negative selection for one particular genome arrangement will affect its segregation is not clear. There is no evidence to suggest that individual mitochondria within a cell are permanently isolated from each other, but it seems rather that they are continually fusing and separating (Honda *et al.* 1966, Jacobs and Lonsdale 1987). If this is the case one might expect selection of individual mitochondria or their alleles within a heteroplasmic cell to be impaired because the whole population will be affected by the products of each genome. Selection would then have to operate between segregants that had arisen randomly. An analogous situation exists in yeast cells where the mitochondrion forms a large reticulate structure; however Backer and Birky (1985) have shown that in non selected cells, mutations to antibiotic resistance become homoplasmic by a combination of random replication and random partitioning, whereas under selection this occurs primarily through intracellular selection of the mutant genomes. The recovery from tobacco cell culture of streptomycin resistant and lincomycin resistant chloroplast mutants (Maliga *et al.* 1975, Cseplo and Maliga 1982) and oligomycin resistant mitochondrial mutants (Aviv and Galun 1988) suggests that it may be possible to recover chloramphenicol resistant transformants.

It was therefore concluded that the results discussed above provide sufficient evidence to justify the use of vectors that could recombine with the resident mitochondrial genome with the expectation that the integrated DNA will then be tolerated and stably propagated. To construct such vectors, sequences from around the *atp9* genes in tobacco and *Petunia* mtDNA (Bland *et al.* 1986, and Young *et al.* 1986) were inserted into the basic mitochondrial transformation vectors to provide them with sequence homology to the tobacco mitochondrial genome.

5.2.1 Construction of Mitochondrial Transformation Vectors Incorporating Sequences from *Petunia* and Tobacco mtDNA.

The mtDNA sequences that were incorporated into the mitochondrial transformation vectors with the aim of promoting homologous recombination are described below.

atp9 -1 from *Petunia hybrida* Mitochondrial DNA.

The *atp9*-1 sequence from *Petunia hybrida* has been described in section 3.1.3.2, section 3.2.3. and Figure 3.14A. Plasmid pATP9-1 which contains the gene on a 1.3 kb *Pst* I to *Bam*HI fragment has been described in section 3.2.3 and Figure 3.14B. The *Petunia* gene (Young *et al.* 1986) and its immediate flanking sequences are highly homologous to the tobacco counterpart (Bland *et al.* 1986, Figure 5.1). This homology comprises a 430 bp sequence present in pATP9-1 extending through the coding region of the gene from about 160 bp upstream of the initiation codon to the end of the cloned fragment 40 bp downstream of the termination codon. This sequence differs from the homologous tobacco sequence at only eleven positions, and was inserted into the mitochondrial transformation vectors to provide a site for homologous recombination with tobacco mtDNA. To do this, the 430 bp sequence was isolated on a larger 1.3 kb *Hin*dIII to *Bam*HI fragment from plasmid pATP9-1 (Figures 3.14 and 5.2). Plasmid pUPS92J was digested at the *Sal*I site downstream from *cat* (Figure 5.2) and treated with calf intestinal phosphatase. After mixing with the *Petunia* sequence, the 5' ends of the fragments were made double stranded with T_4 DNA polymerase, and the resulting molecules ligated and transformed into *E. coli.*

To identify a plasmid containing the *atp9*-1 sequence in pUPS92J, plasmid DNA isolated from 12 chloramphenicol and ampicillin resistant colonies was digested with *Eco*RI. A clone yielding fragments of about 3.0 kb, 1.5 kb and 0.6 kb, which indicate that the *atp9*-1 sequence is present and oriented with similar polarity to *cat*, was further characterised by digestion with *Eco*RI and *Hin*dIII in combination (Figures 5.2 and 5.3). This latter site from the *atp9*-1 fragment is regenerated upon ligation to the *Sal* I site in pUPS92J. The structure was as predicted and the plasmid was designated pJ-PA9 (Figure 5.2).

Although the known homology between the mitochondrial genome of tobacco and pJ-PA9 is confined to 430 bp around *atp9*-1 at the 3' end of its *Petunia* mtDNA insert, the whole of the 1.3 kb fragment was included because it contains all the sequences believed to be responsible for expression of *atp9*-1. Following recombination with the tobacco genome, the promoter region upstream of *atp9* of tobacco will be exchanged for the sequences upstream of *atp9*-1 in pJ-PA9, and it may be important that the tobacco gene is still transcribed. It is hoped that the tobacco gene will function adequately when fused to its *Petunia* counterpart; the high sequence homology between these genes, and the observations that *Petunia* chloroplasts can become established in albino *Nicotiana* cells (Glimelius and Bonnett 1986) and *vice versa* (Pental *et al.* 1986) suggest a degree of functional similarity between the organelles of these genera that may tolerate such an arrangement.

The *atp9* -1 sequence was removed from pJ-PA9 and inserted into the

Figure 5.1

Organisation of the ATP9 Loci of *P. Hybrida* and *N. tabacum* .

The coding regions of the ATP9 genes are represented by the black boxes, and their flanking sequences by the thinner bars. The sites to which transcript termini map are indicated by arrows; at the 5' end the distance in nucleotides from the initiation codon is given, as is the sequence around these sites. Upstream of nucleotide -160 the two sequences diverge precluding alignment. Nucleotide -160 is also the point at which *atp9*-1 diverges from a second *P. hybrida* gene, *atp9*-2. Downstream of this point the two sequences represented in the figure are homologous (greater than 95 %), at least up to the end of the region that has been sequenced. The upstream limit of the homology between the tobacco and *Petunia* genomes coincides with the 5' end of a short sequence that has been found upstream of several plant mitochondrial genes; this is represented by the hatched box, and below its nucleotide sequence is shown and aligned with similar sequences that have been identified upstream mitochondrial genes of other species; the species and gene of origin of each sequence are indicated to their right; the numbering is relative to the start of translation (or the 5' end of mature 26 S rRNA sequence) and refers to the 3' T residue. The sequences surrounding this conserved block show no similarity in the other genes. It has been proposed that this block forms a consensus sequence involved in gene expression (Bland *et al.* 1986, 1987). In support of this, Kennel and Pring (1989) have shown that the 5' ends of the shortest transcripts of the maize *atp6* and T-*urf13* genes map to within a version of this sequence (shown in the figure). A function is not clear, however Mulligan *et al.* (1988b) have shown that primary transcripts of the maize 26S rRNA gene are processed at the 3' end of the conserved block, and the *atp6* and T-*urf13* transcripts that map to this sequence appear also to arise by processing of primary transcripts.

The 3' termini of transcripts of *atp9*-1 (indicated by an arrow) map 195 bp downstream of the termination codon at a sequence similar to that around the 3' termini of three *Oenothera* mitochondrial transcripts (Rothenberg and Hanson 1987b, Schuster *et al.* 1986). A potential stem loop structure that may be involved in generating the 3' terminus is indicated by ❡. The entire sequence of this region is also found to be highly conserved downstream of *atp9* in tobacco. All data is taken from Rothenberg and Hanson (1987b), Bland *et al.* (1986, 1987). E; *Eco*RI site forming part of a potential ribosome binding site. B; *Bam*HI site. P; *Pst*I site.

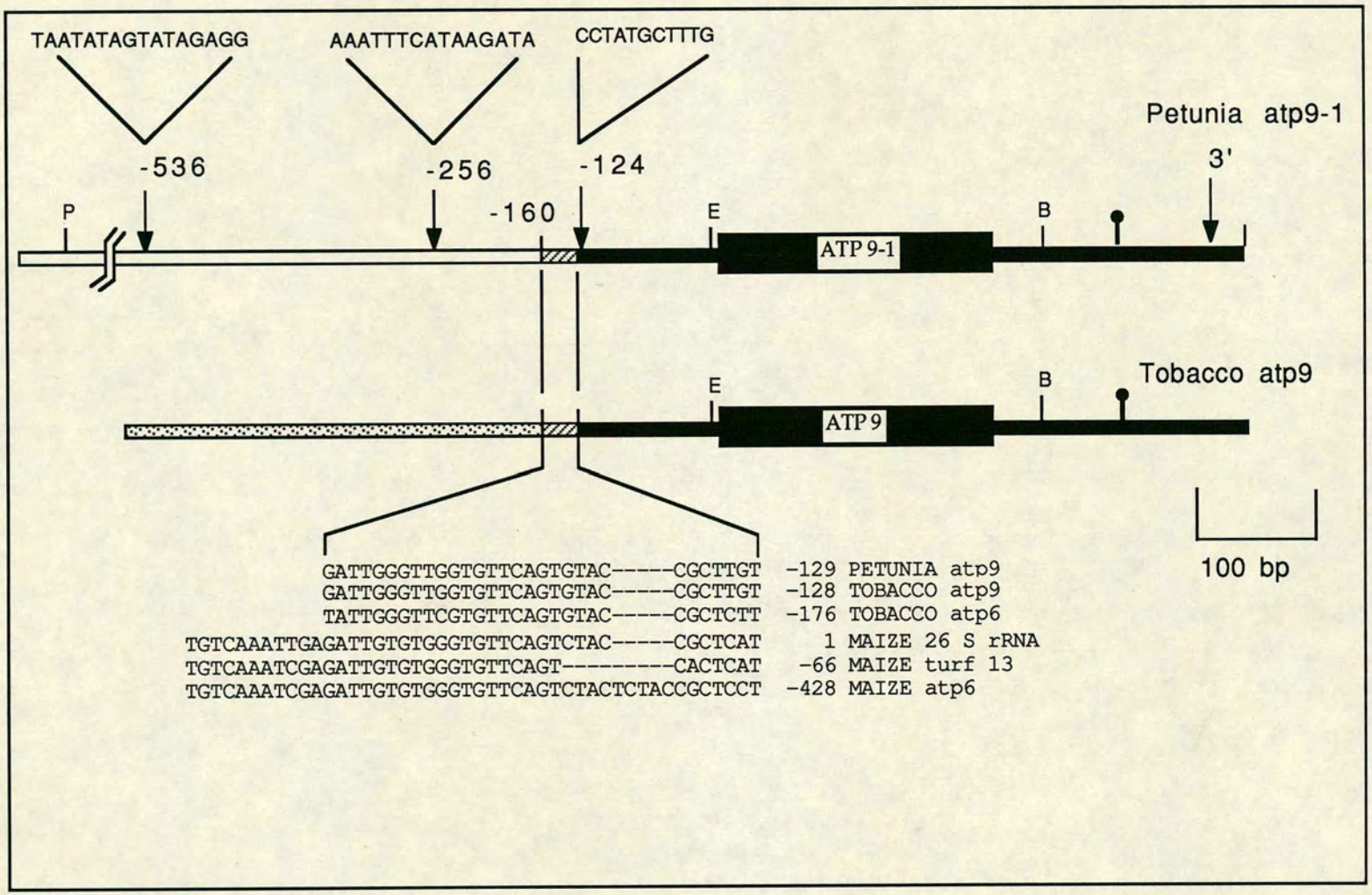

TAATATAGTATAGAGG
AAATTTCATAAGATA
CCTATGCTTTG
Petunia atp9-1
-536
-256
-124
3'
P
-160
E
B
ATP 9-1
Tobacco atp9
E
B
ATP 9
100 bp
GATTGGGTTGGTGTTCAGTGTAC-----CGCTTGT -129 PETUNIA atp9
GATTGGGTTGGTGTTCAGTGTAC-----CGCTTGT -128 TOBACCO atp9
TATTGGGTTCGTGTTCAGTGTAC-----CGCTCTT -176 TOBACCO atp6
TGTCAAATTGAGATTGTGTGGGTGTTCAGTCTAC-----CGCTCAT 1 MAIZE 26 S rRNA
TGTCAAATCGAGATTGTGTGGGTGTTCAGT---------CACTCAT -66 MAIZE turf 13
TGTCAAATCGAGATTGTGTGGGTGTTCAGTCTACTCTACCGCTCCT -428 MAIZE atp6

Figure 5.1

equivalent site of pUPS92E to provide a negative control plasmid (Figure 5.2). To do this, pJ-PA9 was digested with *Hpa*I and *Nde*I which cut respectively within *cat* and pUC9 to release a 2 kb fragment containing the 1.3 kb *Petunia* sequence. Similar digests of pUPS92E were performed but *Pst*I was used in addition to prevent efficient reconstitution of the plasmid during ligation. These multiple digests were mixed, ligated and transformed into *E. coli*. Transformants were screened for low level chloramphenicol resistance to detect plasmids in which *cat* had been regenerated by insertion of the 2 kb *atp9*-1 containing fragment. The presence of this fragment was confirmed in one such plasmid by digestion with *Hin*dIII, and *Eco*RI plus *Bam*HI, and the vector was identified as pUPS92E rather than pUPS92J by digestion with *Bgl*I which cuts asymmetrically in the *coxI* insert (Figure 5.3). This plasmid was designated pE-PA9.

atp9 and Associated Sequences from Tobacco Mt DNA.

The genes encoding the α subunit of the F1 ATP synthase and subunits 9 and 6 of the Fo portion have been isolated from tobacco mitochondrial DNA (Chaumont *et al.* 1988 and Bland *et al.* 1986, 1987 respectively). A clone of *atp9* was the only one of these that was available when this work was undertaken and was used to provide the vectors with a site for homologous recombination. Plasmid pATP S13 contains a 2.9 kb *Pst*I fragment from *N. tabacum* encoding *atp9* in addition to 1.3 kb upstream of the initiation codon, and downstream of the stop codon, an open reading frame encoding a product with homology to the *E. coli* ribosomal protein S13 and a reading frame whose predicted product is homologous to an internal portion of NAD1 (subunit 1 of the NADH Ubiquinone Oxidoreductase, Figure 5.4A). These three reading frames are cotranscribed to produce a complex set of messages of between 600 and 6,000 nucleotides. The two smallest ones, of 600 and 1,300 nucleotides hybridise only to sequence from *atp9* and may be processing products (Bland *et al.* 1986). The origin of the transcripts was not determined in the published work, but is discussed in section 5.2.2.

The function of the other two reading frames is unclear. Hybridisation and sequencing studies have shown highly homologous S13 reading frames to be present and transcribed in maize (Bland *et al.* 1986) and *Oenothera* (Schuster and Brennicke 1987c). The S13 ORF does not hybridise to pea or bean mitochondrial DNA, and in wheat the gene is present, but appears not to be transcribed (Bonen 1987). Whether these plants, and perhaps the others, have active S13 genes in their nuclei is not known.

The significance of the reading frame that may encode NAD 1 is equally unclear. It is found downstream of S13 in tobacco, and maize, and hybridises to

Figure 5.2

Construction of Plasmids Containing the ATP9-1 Gene of *P. hybrida* Providing Sequence Homology to the Tobacco Mitochondrial Genome.

The coding and flanking sequences of the *P. hybrida* ATP9-1 gene that were inserted into the basic transformation vectors were isolated on a 1.3 kb *Hin*dIII (H) to *Bam*HI (B) DNA fragment from pATP9-1. This fragment is illustrated at the top of the figure. The coding sequence of *atp9*-1 is represented by the box, and its direction of transcription is indicated by the arrow; the region of known homology to tobacco mitochondrial DNA is in black. The mitochondrial transformation vectors pUPS92J, pUPS92E, pSCOX92O and the derivatives containing the *atp9*-1 sequence are shown below this fragment. The bacterial vector sequences have been omitted for simplicity, but are shown for each of the component plasmids in Figures 3.9 and 3.14. pUPS92J is drawn as in Figure 3.9B, except that the *Nde*I site in the vector at the 3' end of *cat* has been included.

Plasmid pJ-PA9 was constructed by inserting the *Hin*dIII to *Bam*HI fragment of pATP9-1 into the *Sal*I site (S) of pUPS92J as described in the text. Only the *Hin*dIII site remains intact after ligation (the other recognition sites which are lost are shown in brackets). Insertion of *atp9*-1 in this orientation creates a new *Eco*RI (E) fragment of 1.5 kb and a *Hin*dIII fragment of 1.3 kb (see Figure 5.3).

Plasmid pE-PA9 was constructed by replacing the *Hpa*I (Hp) to *Nde*I fragment of pUPS92E with that of pJ-PA9 as described in the text. The asymmetric *Bgl*I site (Bg) has been indicated.

Recently an equivalent pSCOX92O derivative has been assembled by inserting the *Hin*dIII fragment of pE-PA9 as shown in the diagram (symbols are the same as in Figure 3.9); this plasmid has not been used for plant transformation but is available for future use.

Figure 5. 2

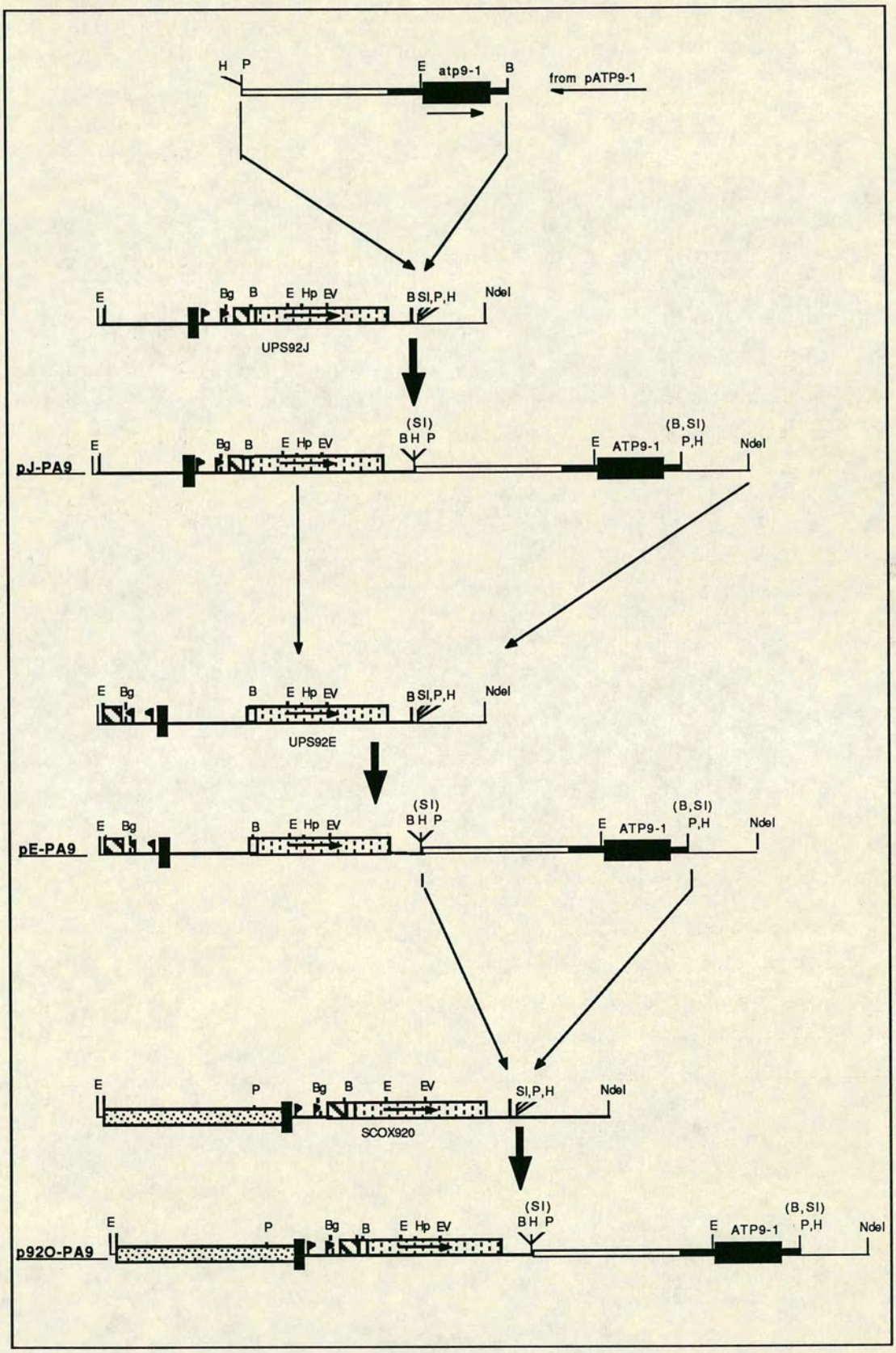

H P
E atp9-1 B
from pATP9-1
E Bg B E Hp EV B SI,P,H NdeI
UPS92J
(SI) B H P
(B,SI) P,H
pJ-PA9
E ATP9-1
NdeI
E Bg B E Hp EV B SI,P,H NdeI
UPS92E
pE-PA9
E P Bg B E EV SI,P,H NdeI
SCOX920
p92O-PA9

Figure 5.3

Restriction Endonuclease Digestion Analysis of pJ-PA9 and pE-PA9.

Plasmid DNA was digested with restriction endonucleases, products were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed under U.V. illumination. The products of *Hin*dIII digestion of bacteriophage λ DNA and of *Hae*III digestion of bacteriophage ϕX174 were used as DNA size markers, and the approximate size of each band is shown at the right of each gel.

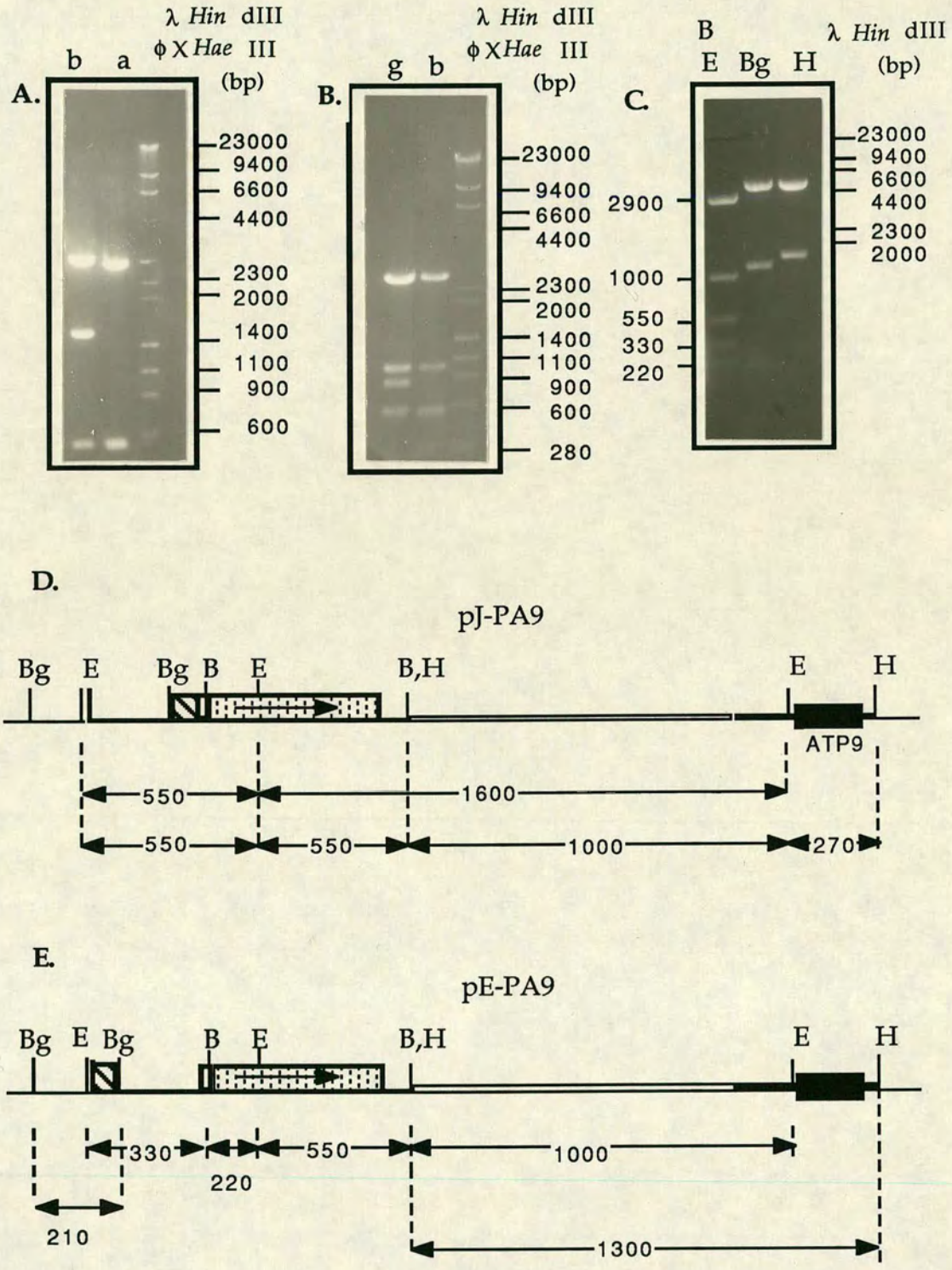
A. *Eco* RI digest of pJ-PA9 (lane b) containing the 1.3 kb ATP9-1 insert at the *Sal*I site of pUPS92J, and a similar digest of pUPS92J religated without insertion of the ATP9-1 sequence (lane a). The origin and approximate size of the fragments expected to result from digestion of pJ-PA9 with *Eco*RI are shown in **D**.

B. pJ-PA9 (lane b) and a second clone containing the 1,3 kb ATP9-1 sequence in the reverse orientation (lane g) digested with a combination of *Eco*RI and *Hin*dIII. The origin and approximate size of the fragments expected to result from digestion of pJ-PA9 with these enzymes are shown in **D**.

C. Plasmid pE-PA9 was digested with *Hin*dIII (H), *Bgl*I (Bg), or a combination of *Eco*RI (E) and *Bam*HI (B) as indicated at the top of each lane. The digestion products observed were consistent with the expected sizes shown in **E**; the fragments corresponding to each of the expected *Eco*RI and *Bam*HI double digestion products are indicated on the left.

D and **E** are schematic diagrams of pJ-PA9 and pE-PA9 showing the size and origin of the expected restriction endonuclease fragments. They are drawn similarly to the diagrams of these plasmids in Figure 5.2. except that the *Bgl*I site approximately 150 bp upstream of the COXI gene promoter sequence has been included. Digestion of pE-PA9 with this enzyme produced a fragment of approximately 210 bp as expected, whereas a similar digest of pJ-PA9 would have produced a fragment of approximately 400 bp. Digestion of each plasmid with *Hin*dIII confirmed the presence of the regenerated *Hin*dIII recognition site immediately downstream of the CAT gene. *Eco*RI digestion of pJ-PA9 showed the orientation of the 1.3 kb ATP9-1 insert. Symbols are identical to those used in Figure 5.2.

Figure 5.3


A.
b a
λ Hin dIII
ϕX Hae III
(bp)
23000
9400
6600
4400
2300
2000
1400
1100
900
600
B.
g b
λ Hin dIII
ϕX Hae III
(bp)
23000
9400
6600
4400
2300
2000
1400
1100
900
600
280
C.
B
E Bg H
λ Hin dIII
(bp)
23000
9400
6600
4400
2300
2000
2900
1000
550
330
220
D.
pJ-PA9
Bg E Bg B E B,H E H
ATP9
550
1600
550
550
1000
270
E.
pE-PA9
Bg E Bg B E B,H E H
330
550
1000
220
210
1300

mtDNA from *Oenothera* and *B. campestris*, however in the latter species it appears not to be transcribed. The product predicted from the reading frame is homologous to the central region and carboxy terminus of other NAD1 proteins; however the similarity ends abruptly between 122 and 128 residues from the N-terminus of these proteins (Figure 5.5). Upstream of this divergence point, the reading frame in maize mtDNA encodes a product with poor homology to the other NAD1 proteins, and the one in tobacco mtDNA extends for only thirteen codons, none of which encode methionine to initiate translation (Bland *et al.* 1986). A similar situation seems to exist in watermelon where Stern *et al.* (1986) have found a truncated sequence similar to that in tobacco, and proposed that the missing N-terminal region is encoded on an upstream reading frame of 300 bp which could form a separate exon. The DNA sequence of this putative exon was compared with the corresponding sequence upstream of the putative NAD1 genes of tobacco and maize. This revealed that although the sequence is present in the latter species, a number of minor nucleotide differences exist which alter the reading frame and introduce premature termination codons. The significance of this putative exon is thus questionable in all three species. Bland *et al.* (1986) were unable to find any portion of the entire 2.9 kb insert of pATP S13 that could encode the missing N-terminal sequence, or any homology to the 3' portion of the reading frame elsewhere in the tobacco mitochondrial genome. This leaves the location of the missing *nad1* sequences undiscovered, and the significance of the *nad1* homologous sequences unclear, making it particularly difficult to assess the likely effect of inserting the selectable marker in this region.

To insert this tobacco mtDNA sequence (S13) into the mitochondrial transformation vectors, pATP-S13 was digested with *Pst*I to release the 2.9 kb mtDNA fragment. This was inserted into pUPS92J and pUPS92E at their unique *Pst*I sites (Figure 5.4). Recombinant plasmids were characterised by digestion with *Eco*RI, *Pst*I, and *Eco*RI with *Bam*HI (Figure 5.6A) and clones bearing the insert were called p 92J-S13 and p92E-S13. In these plasmids, *cat* and *atp9* are oriented with similar polarity.

The (S13) fragment from tobacco mtDNA fragment could not simply be inserted at the equivalent *Pst* I site in pSCOX92O because this plasmid contains a second *Pst*I site within the SCOX promoter fragment from maize mtDNA (Figure 3.9). Instead, a 2.9 kb *Sal*I to *Hin*dIII fragment containing the entire S13 fragment was removed from pATP-S13 and was inserted into the pSCOX92O polylinker downstream of *cat*. After ligation and transformation, the desired plasmid was identified as above (Figure 5.4 and 5.6 A). It was called p92O-S13.

The mitochondrial transformation vector pAPcat1 possesses a single *Pst* I site at the 5' end of its *Petunia atp9*-1 sequence (Figure 3.14). This plasmid and pATP-S13 were digested with *Pst*I, mixed, ligated and after transformation derivatives of pAPcat1 carrying the S13 fragment of pATP-S13 were recovered.

Figure 5.4

Construction of Mitochondrial Transformation Vectors Containing Sequence from Tobacco Mt DNA that Provide Potential Sites for Homologous Recombination with the Tobacco Mitochondrial Genome and for Generation of 3' Termini of CAT Gene Transcripts.

A. The polylinker cloning region [*Eco*RI (E) to *Hin*dIII (H)] of plasmid pATP-S13 that contains a 2.9 kb *Pst*I (P) fragment from *N. tabacum* mitochondrial DNA is indicated at the top of the panel. The 2.9 kb *Pst*I fragment is shown by the heavy line; flanking pUC8/9 vector sequences are represented by thin lines. The three reading frames with homology to known proteins are indicated, but described in detail in Figure 5.5 and in the text; arrows indicate the direction of transcription. The stem loop that may be involved in transcription termination is indicated by ♀.

This tobacco mtDNA sequence was inserted into each of the mitochondrial transformation vectors pUPS92J, pUPS92E and pSCOX92O using one of two cloning strategies. Plasmids p92J-S13 and p92E-S13 were constructed by inserting this *Pst* I fragment into the unique *Pst* I site of pUPS92J and pUPS92E respectively. To make the equivalent pSCOX92O derivative the *Hin*dIII and *Sal*I sites flanking the mitochondrial DNA sequence in pATP-S13 and similar sites downstream of *cat* in pSCOX92O were used.

B. The region of the 2.9 kb *Pst* I fragment downstream of the ATP9 coding region may contain a transcription termination signal or transcript processing site (see text). To place the putative termination signal downstream of *cat*, a 1.4 kb *Bam*HI to *Hin*dIII fragment from pATP-S13 was used; this is drawn at the top of the panel, and is labelled 'T13'. Plasmids p92J-T13, p92E-T13 and p92O-T13 were constructed in a two step process described in the text, and each contains the T13 sequence inserted between the *Bam*HI and *Hin*dIII sites downstream of *cat* in pUPS92J, pUPS92E and pSCOX92O respectively.

E, B, Sl,P and H indicate recognition sites for *Eco*RI, *Bam*HI, *Sal*I, *Pst*I and *Hin*dIII respectively.

Figure 5.4

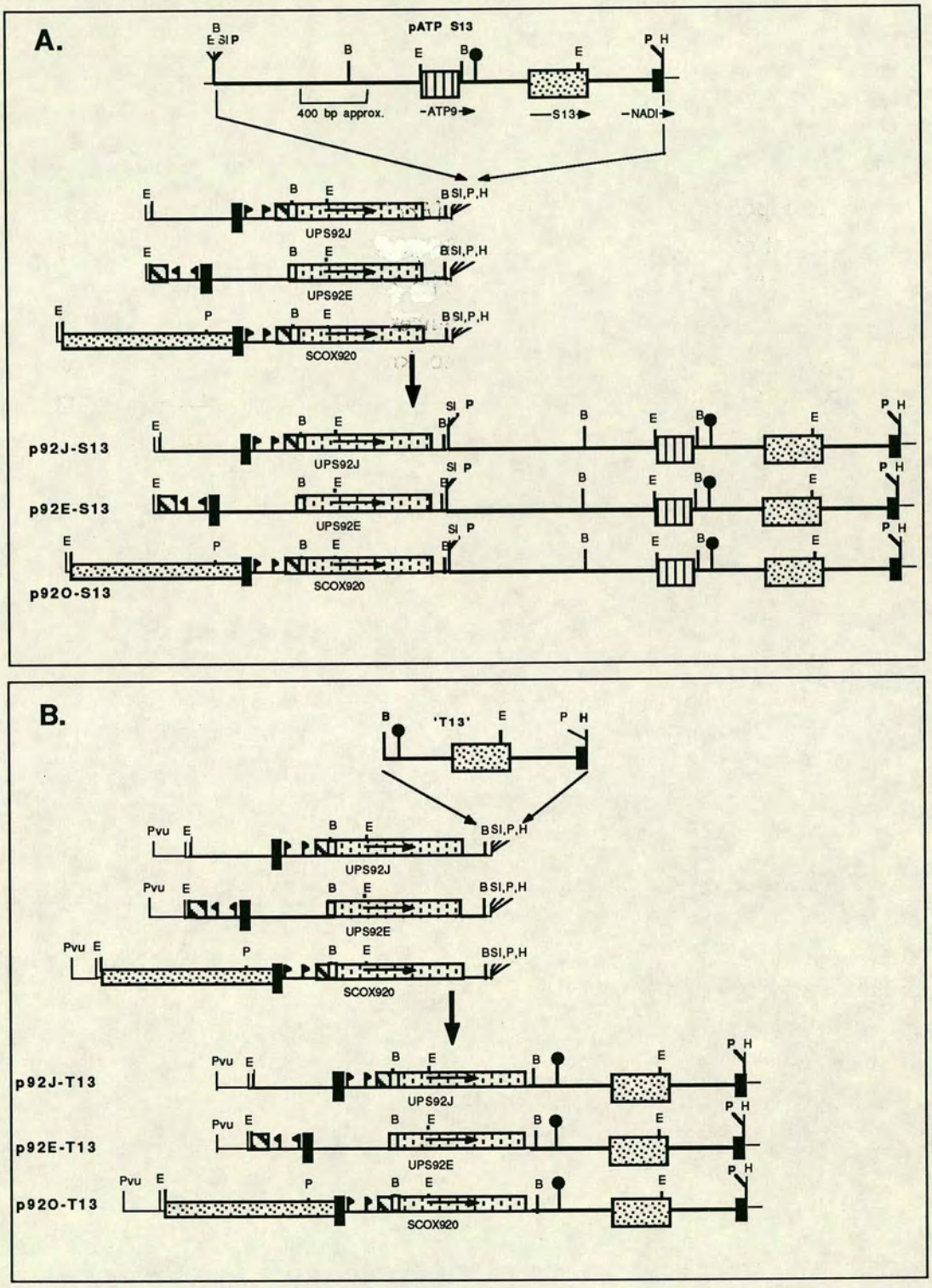

A.
pATP S13
400 bp approx.
-ATP9-
-S13-
-NADI-
UPS92J
UPS92E
SCOX920
p92J-S13
p92E-S13
p92O-S13
B.
'T13'
p92J-T13
p92E-T13
p92O-T13

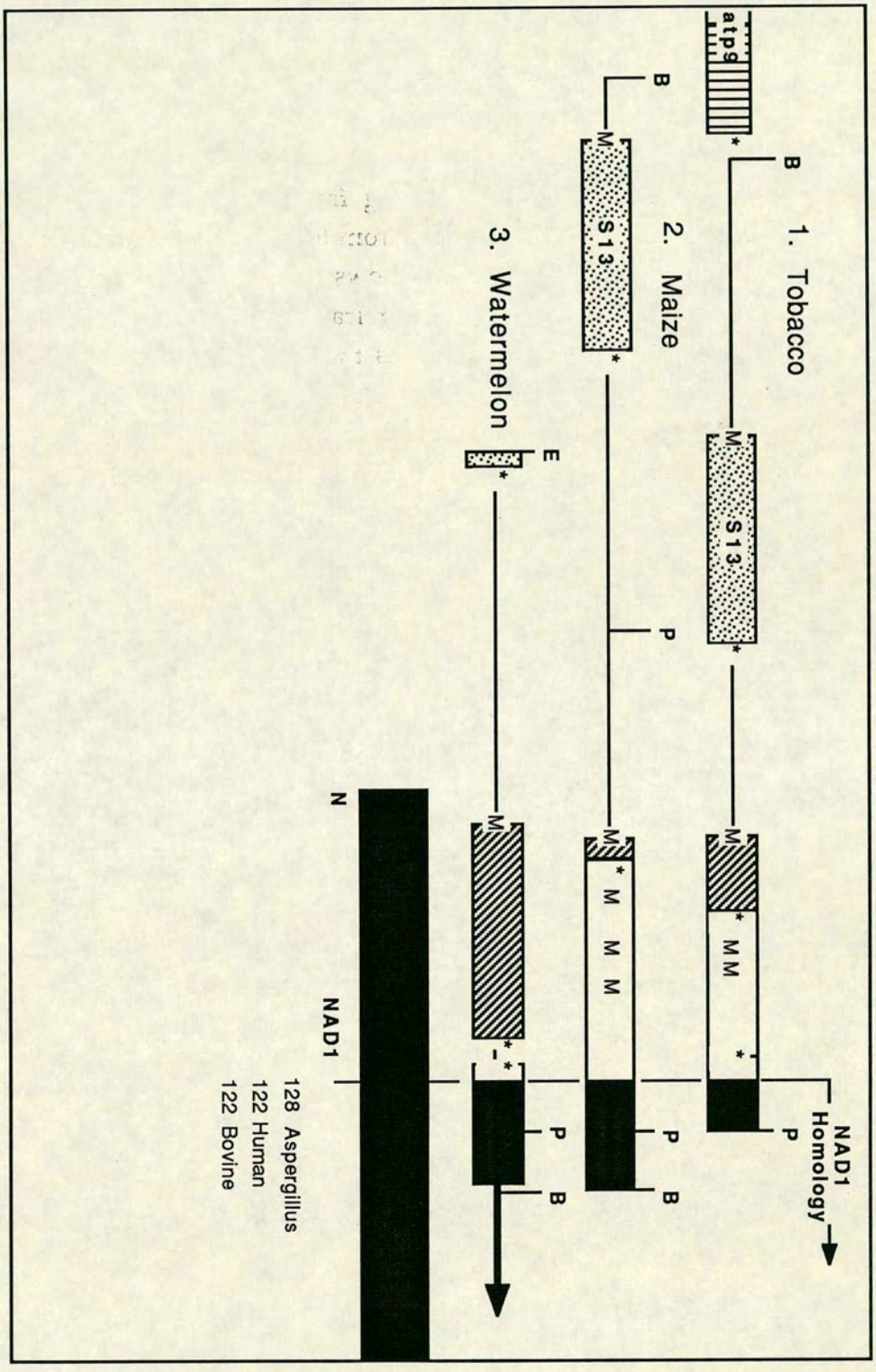
Figure 5. 5

Organisation of the *nadI* locus in Several Higher Plant Mitochondrial Genomes.

A reading frame that potentially encodes a protein homologous to part of NAD1 has been isolated from mtDNA of tobacco, maize and watermelon and its nucleotide sequence has been determined (Bland *et al.* 1986, Stern *et al.* 1986). The genomic organisation of this reading frame in each species is shown; 1. shows part of a 2.9 kb *Pst*I (**P**) fragment from tobacco (see figure 5.4), 2. shows a *Bam*HI (**B**) fragment from maize, and 3. shows part of an *Eco*RI (**E**) fragment from watermelon. Boxes representing reading frames encoding *atp9* and *rps13* are labelled; regions of other reading frames that are homologous to NAD1 from *Aspergillus*, human and bovine mtDNA are in black. Initiation (**M**) and termination (✳) codons are indicated in each reading frame. The black box at the bottom of the figure represents NAD1 from the three species indicated; **N** indicates its N-terminus and the numbers refer to the amino acid residues at which homology between these proteins and all three plant mitochondrial reading frames begins. The homology extends at least to the end of the tobacco and maize restriction fragments; in watermelon the cloned fragment probably includes the remainder of the C-terminal coding sequence, but is probably split by one or more introns (heavy arrow) and is not shown in its entirety. The maize reading frame continues for 101 residues upstream of the NAD1 homologous region and includes several potential initiation codons. However the predicted translation product of this extended reading frame shows poor conservation in comparison with the N-terminal region of the mammalian and fungal NAD1. The tobacco and watermelon reading frames terminate respectively only 13 and 9 residues upstream, of the NAD1 homologous region, and these short sequences do not contain initiation codons.

It has been proposed that in watermelon an upstream open reading frame (ORF, hatched box) forms a separate exon encoding the N-terminal portion of NAD1, though its predicted protein product shares only 22 % homology to other NAD1 proteins. The sequences upstream of the NAD1 homologous reading frame are similar (about 90%) in each plant species except for several deletions or insertions; thus although most of the sequence of the upstream ORF is present in tobacco and maize it terminates after 38 and 7 residues respectively (hatched boxes). A watermelon sequence homologous to the 3' end of *rps13* is shown by stippling.

Figure 5.5


1. Tobacco
2. Maize
3. Watermelon
atpg
S13
M
B
E
P
NAD1
Homology
N
128 Aspergillus
122 Human
122 Bovine

These were mapped by digestion with restriction endonucleases, and clones with the tobacco *atp9* coding sequence oriented with similar and opposite polarity to *cat* were called pAP S13 and pAPS13.9 respectively (Figure 5.6 A).

It is predicted that following recombination between these plasmids and the tobacco mitochondrial genome, sequences upstream of the resident *atp9* will be replaced by the 1.3 kb portion of S13 upstream of *atp9* in the transformation vectors. As mentioned above, it has not been directly shown that this sequence includes the necessary expression signals but by analogy with other plant mitochondrial genes it is likely to do so (section 3.1.3).

Bland *et al.* (1985) have reported that tobacco mtDNA contains a sequence homologous to the chloroplast 23S rRNA gene. Attempts are being made to obtain this sequence and to use it for homologous recombination at a genomic site that will probably not affect mitochondrial function.

5.2.2 Construction of Mitochondrial Transformation Vectors Containing A Potential Transcription Terminator or 3' Processing Site.

The mitochondrial transformation vectors described above contain specific sequences to initiate transcription, but attention has not yet been paid to its termination.The requirement for initiation of transcription in gene expression is obvious. The importance of its termination or of processing the mRNA beyond the coding sequence is less clear. In yeast mitochondria, a conserved nucleotide sequence is found downstream of many genes, and appears to be involved in generating the 3' end of the transcript by processing (Osinga *et al.* 1984a). Stern and Gruissem (1987) have identified inverted repeat sequences near the 3' termini of chloroplast genes which appear to be involved in processing a longer precursor and then stabilising the mature mRNA.

All that is known about transcription termination in plant mitochondrial genes is that their mRNA seems not to be polyadenylated (Schuster *et al.* 1986, Hiesel and Brennicke 1987a). Inverted repeats located downstream of several maize and *Oenothera* genes have been implicated in generating the 3' ends of transcripts either by processing or by termination of transcription (Schuster *et al.* 1986), but are not found in all mitochondrial genes isolated from even these genera (for example Schuster *et al.* 1986, Hiesel *et al.* 1987b).

It is not known whether proper expression of mitochondrial genes is dependent upon the 3' termini of their transcripts being located at a particular point; similarly, it is not clear what the consequences may be if termination of transcription or transcript processing was to occur at random points, or even not at all. In the absence of direct evidence, the potential requirement for a particular

Figure 5.6 A

Restriction Endonuclease Digestion Analysis of pAP-S13, p92J-S13, p92E-S13 and p92O-S13.

Plasmid DNA was digested with one or a combination of the restriction endonucleases *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H) and *Pst*I (P) as indicated at the top of each lane. The products were separated by electrophoresis through an agarose gel, stained with ethidium bromide and photographed under U.V. illumination. DNA size markers were either *Ava*II (Av) or *Hin*dIII digests of bacteriophage λ DNA, and the approximate size of each band in base pairs is indicated adjacent to each gel.

A. Digestion products of pAP-S13.

B. Digestion products of p92J-S13 and p92O-S13.

C. Digestion products of p92E-S13.

The origin and approximate size in base pairs of the expected products of each digestion are shown below the gels. The symbols are the same as those used in Figure 5.4, except for AP-S13 where the sequence derived from the *P. hybrida* ATP9-1 promoter region is indicated by the white box containing black triangles. In each case, the *Eco*RI digest shows the orientation of the S13 insert.

Figure 5.6a

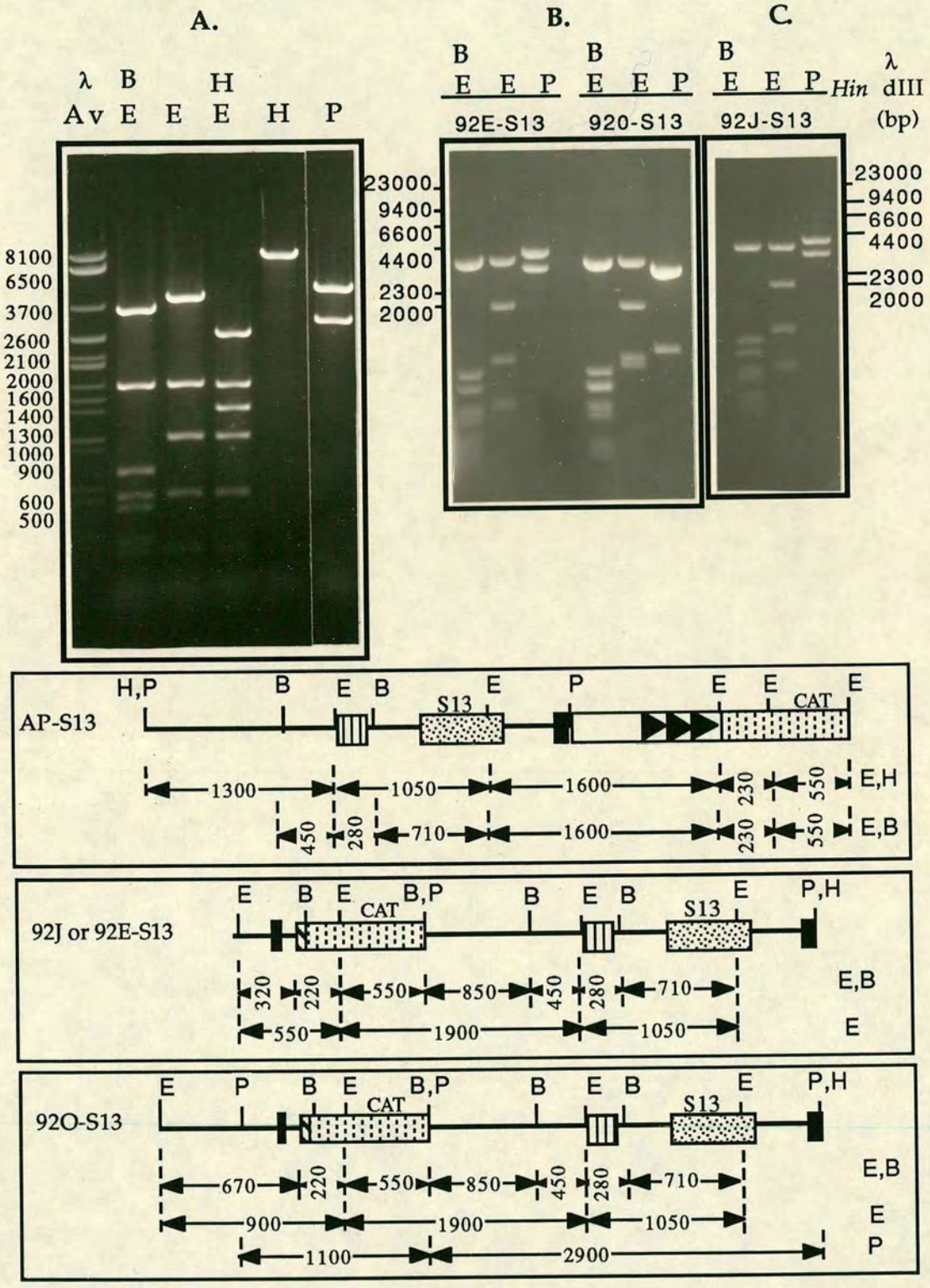
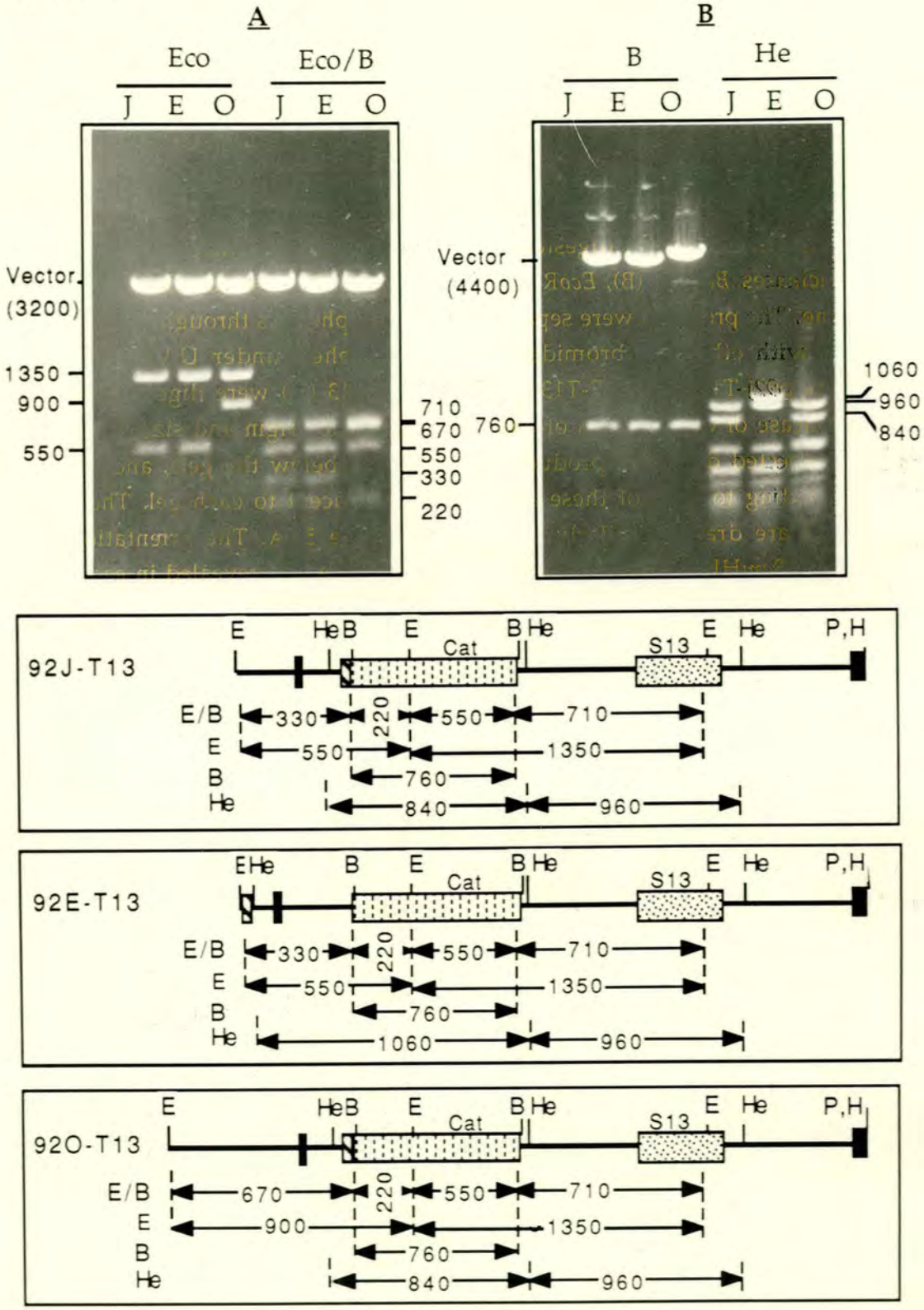

A.
B.
C.
λ B H
Av E E E H P
8100
6500
3700
2600
2100
2000
1600
1400
1300
1000
900
600
500
B B B
E E P E E P E E P Hin λ dIII
92E-S13 920-S13 92J-S13 (bp)
23000
9400
6600
4400
2300
2000
AP-S13
H,P B E B S13 E P E E CAT E
1300 1050 1600 230 550 E,H
450 280 710 1600 230 550 E,B
92J or 92E-S13
E B E CAT B,P B E B S13 E P,H
320 220 550 850 450 280 710 E,B
550 1900 1050 E
92O-S13
E P B E CAT B,P B E B S13 E P,H
670 220 550 850 450 280 710 E,B
900 1900 1050 E
1100 2900 P

Figure 5.6 B

Restriction Endonuclease Digestion Analysis of p92J-T13, p92O-T13, and p92E-T13.

Plasmid DNA was digested with one or a combination of the restriction endonucleases *Bam*HI (B), *Eco*RI (Eco) and *Hae*III (He) as indicated at the top of each lane. The products were separated by electrophoresis through an agarose gel, stained with ethidium bromide and photographed under U.V. illumination. Plasmids p92J-T13 (J), p92E-T13 (E) and p92O-T13 (O) were digested with each endonuclease or combination of endonucleases. The origin and size in base pairs of the expected digestion products are indicated below the gels, and the bands corresponding to each of these are indicated adjacent to each gel. The plasmid diagrams are drawn similarly to those in Figure 5.6A. The orientation of the reinserted *Bam*HI fragment containing the CAT gene was revealed in each case by digestion with *Eco*RI.

Figure 5.6b


A
Eco
Eco/B
J E O J E O
Vector
(3200)
1350
900
550
710
670
550
330
220
B
B
He
J E O J E O
Vector
(4400)
760
1060
960
840
92J-T13
92E-T13
92O-T13
E
He B
E
Cat
B He
S13
E
He
P,H
E/B
E
B
He
330
220
550
710
550
1350
760
840
960
1060
670
900

3' structure cannot be dismissed.

Downstream of *atp9* in tobacco, there is an inverted repeat similar to those postulated by Schuster *et al.* (1986), but no transcript mapping has been done to assess its significance (Bland *et al.* 1986). An analogous structure occurs 3' of *atp9* from maize and two different ATP9 genes from *Petunia* (Bland *et al.* 1986, Rothenberg and Hanson 1987), and in the latter case the 3' ends of the major transcripts have been mapped to about 80 bp beyond these structures. This result is interesting because the sequence downstream of *atp9* in tobacco is almost identical to that downstream of *atp9*-1 from *Petunia* up to and beyond the site of the transcript terminus and on to the end of the published sequence. If this sequence functions as a terminator in *Petunia* , it may do so in tobacco. As shown in Figure 5.1. the similarity between the two *Petunia* ATP9 genes and their tobacco homologue extends to a point just upstream from the 5' end of their shortest and most abundant transcript. If the same sequences were functional in generating the 5' and 3' termini of *atp9* transcripts in both genera, the tobacco gene would be predicted to produce a transcript of about 550 nucleotides. From Northern blot analysis, Bland *et al.* (1986) estimated the most abundant *atp9* transcript to be 600 nucleotides in length. As in *Petunia* this was the shortest transcript, and it showed no hybridisation to a region 200 bp beyond the proposed 3' terminus. Most *atp9*-1 transcripts are reported to terminate at this site, but this is not so for the divergent *atp9*-2 (Rothenberg and Hanson 1987), and also in tobacco, Northern blots show that there are a significant number of transcripts that extend through the S13 reading frame and into the NAD1 reading frame downstream. The differences in the apparent efficiency with which transcript termini are generated by these related sequences may be attributable to differential stability of the 3' portion of the RNA, or to sequence differences in the signals that generate the termini. It was decided to insert this sequence from the tobacco mitochondrial genome into the transformation vectors in order to provide a potential transcript termination site and a sequence for homologous recombination on a single DNA fragment.

The sequences proposed to be involved in generating the transcript termini are located on the 1.4 kb *Bam*HI to *Hin*dIII fragment of plasmid pATP-S13 begining 34 bp 3' to *atp9* and ending in the NAD1 homologous reading frame (Figures 5.1, 5.4A and B, and Bland *et al.* 1986, Schuster *et al.* 1986, Rothenberg and Hanson 1987b). This fragment, designated T13, was introduced into the basic mitochondrial transformation vectors in a two step cloning strategy. In the first step that generated intermediate plasmids, T13 was isolated from pATP-S13 and inserted into the gel purified vectors pUPS92J and pUPS92E (Figure 5.4 B) which had been cut with *Bam*HI and *Hin*dIII. Recombinant plasmids carrying the T13 insert were called pT13-J and pT13-E (not shown); they retain the mitochondrial promoter sequence (UPS), but have lost the CAT gene as a result of the *Bam*HI

digestion used in their construction. An equivalent plasmid called pT13-S was constructed by digesting p92O-S13 (Figure 5.4 A) with *Bam*HI and religating the vector fragment. Plasmid pT13-S like the pT13-J and pT13-E has lost the CAT gene owing to the *Bam*HI digestion. Therefore, the fragment encoding *cat* was isolated from a *Bam*HI digest of pUPS92J for reinsertion into the *Bam*HI site of these three plasmids at the junction between the promoter and T13 sequences. Ligation products were transformed into NM522. Ampicillin resistant transformants were screened for resistance to 6 µg/ml chloramphenicol, and their plasmid DNA characterised with *Bam*HI, *Bam*HI with *Eco*RI, *Eco*RI, and *Hae* III (Figure 5.6 B). Plasmids with *cat* inserted in the desired orientation were called p92-T13, p92E-T13 and p92O-T13 (Figure 5.4 B). In these plasmids, the proposed *atp9* transcript termination site is situated 250 bp downstream from the termination codon of *cat* .

Recombination between these plasmids and the mitochondrial genome will not alter the sequence downstream of *atp9* within 1.4 kb of its termination codon, and so its expression is unlikely to be affected. If, however, expression of the S13 and NAD1 homologous sequences is required, then in the recombined allele this will probably be dependent upon cotranscription with *cat* from the maize *coxI* promoter instead of cotranscription with *atp9* from its promoter. Whether or not this situation would be tolerable in enough genomes to provide chloramphenicol resistance is discussed in Chapter 8.

5.3 Independent Mitochondrial Replicons for maintenance of the Transferred sequences in Plant Mitochondria.

A strategy that presents an alternative to integration of transforming DNA into the main genome is to provide the transformation vectors with sequences that will allow them to replicate independently of the main genome. Mitochondrial DNA preparations frequently contain circular DNA molecules, usually of between 1 and 2 kb. Such minicircles are generally assumed to be capable of independent replication because they show no hybridisation to the high molecular weight genomic DNA, and because they preferentially incorporate radioactive deoxynucleotides (Smith *et al.* 1987). In addition electron microscopic analysis of preparations of *Vicia faba* mitochondrial DNA has revealed putative replication intermediates of such minicircular DNA, and has allowed the replication origins to be mapped (Wahleitner and Wolstenholme 1987, 1988). Minicircles have been cloned and sequenced from *Vicia faba* (Wahleithener and Wolstenholme 1987), sugar beet (Hansen and Marker 1984, Thomas 1986), and maize (Ludwig *et al.* 1985, Smith and Pring 1987), but not from tobacco. Kemble

et al. (1988) have shown that following fusion of *Brassica* protoplasts mitochondrial minicircles can be stably transferred from one cell line to cell lines that do not normally contain them. If a selectable marker were to be incorporated into such a minicircle and the capacity for independent replication was preserved, problems associated with integration and segregation of transforming DNA following transformation could be avoided.

The difficulty of achieving this in practice is that despite the frequent observation that such minicircles encode transcripts and show homologies to other replicons nothing is known of the requirements for their replication. It would be advantageous to know if they would replicate in any other than their species of origin, and also whether extra DNA sequence could be included, and if so, at which site this could be inserted.

This alternative has not been so actively pursued because of these difficulties, but potential replicons from maize, sugarbeet, and *Oenothera* have been obtained, and their insertion as dimers into the mitochondrial vectors has been initiated in an attempt to construct an active replicon.

An *in vitro* replication assay for mitochondrial molecules similar to that for chloroplast genomes (de Haas *et al.* 1988) could potentially aid the evaluation of these constructs.

5.4 Conclusion.

In this chapter I have discussed the requirement for provision of specific sequences to maintain the transforming DNA in mitochondria and ways in which this might be achieved. Insertion of the selectable marker gene into the mitochondrial genome was chosen as the most suitable way of ensuring maintenance of the transferred DNA. A discussion of the current understanding of recombination and inheritance of plant mitochondrial DNA has been given and used to justify this approach. Several plasmids were constructed that could potentially recombine with the tobacco mitochondrial genome via regions of homology, and these have been described. In one set of such plasmids a potential transcription terminator has been included.

CHAPTER 6.

SELECTION AND SCREENING OF CHLORAMPHENICOL RESISTANT PLANT CELLS.

6.1 Introduction.

The advantage of an efficient selection for the products of potentially rare events like mitochondrial transformation was explained in Chapter 3. The most efficient nuclear gene transfer systems developed so far have involved transformation of protoplasts with either *A. tumefaciens* (for example Zambryski *et al.* 1983b) or purified DNA (Shillito *et al.* 1985). De Block *et al.* (1984) have used chloramphenicol to select protoplast derived calli transformed with the CAT gene. They found that for best results, calli had to be grown in liquid medium in the absence of chloramphenicol and had then to be transferred to solid media containing chloramphenicol. Transformants showed poor resistance and could not be maintained for more than 3 months on chloramphenicol.

The most common and convenient way to culture large numbers of protoplasts (more than $5x10^5$) and to select transformants is using the agarose bead system (Figure 6.4A) which is described in Materials and Methods (2.3.4.2). However De Block *et al.* (1984) were unable efficiently to select chloramphenicol resistant transformants this way. Therefore my initial aim was to establish a more convenient and efficient selection system based on the selection of protoplsts in the agarose bead culture system.

6.2. Chloramphenicol Sensitivity of Regenerating Protoplasts.

Protoplasts start to regenerate a cell wall and to divide within a few days of isolation provided they are cultured in a suitable medium. To examine the effect of chloramphenicol on this process, protoplasts were subjected to mock transformation and regenerated in the presence of different concentrations of chloramphenicol. Protoplasts were prepared from *N. tabacum* cv. Petit Havana SR1, and put through the PEG-calcium nitrate transformation procedure, but in

the absence of DNA. After a week, the sample was embedded in agarose as described in section 2.3.4.2, and divided into six equal portions which were plated out in medium with 0, 10, 20, 30, 40 or 50 µg chloramphenicol/ml. Protoplasts were cultured as normal for six weeks, and the growth of calli subjected to each treatment was assessed (Figure 6.1). Although the inclusion of chloramphenicol reduced growth rates at all concentrations, it stopped growth at 40 µg/ml. This value is higher than that reported by De Block *et al.* (1984), but similar to that found by Pietrzak *et al.* (1986), and was chosen as the lower limit of stringent selection. Calli have been cultured for longer periods under similar experimental conditions without growth being observed at chloramphenicol concentrations above 30 µg/ml, but continuing at or below this concentration (not shown). The object of these experiments was to determine the least stringent selection conditions that would prevent growth of the vast majority of the untransformed calli. It was hoped that the use of these conditions would allow recovery of calli that express the lowest levels of resistance.

6.3. Selection of Resistant Calli after Protoplast Transformation.

6.3.1 Selection at a Single Chloramphenicol Concentration.

In order to develop a procedure for selection of mitochondrial transformants using chloramphenicol, selection of chloramphenicol resistant nuclear transformants was used as a model system. Plasmid pCAP212 had been shown to express *cat* in plant cells (Velten and Schell 1985 and section 4.4.1) and was initially used to develop a procedure for selection with chloramphenicol. This plasmid also expresses *nptII* in plant cells, and has been used to select kanamycin resistant transformants (Velten and Schell 1985); thus, in this series of experiments, after transformation with pCAP212, some of the transformed protoplasts were also selected with kanamycin as a control for transformation. In addition, the recovery of transformants from the same initial transformation experiment using each antibiotic was compared. The two selectable genes in pCAP212 are expressed from promoters, translation initiation sites and polyadenylation sites that are different, so a rigorous comparison of the efficiency of selection with the corresponding antibiotics was not possible. However, the number of kanamycin resistant transformants recovered did provide a lower limit for the number of potential chloramphenicol resistant transformants available for recovery and thus allowed an estimate of the maximal efficiency with which transformants were recovered with chloramphenicol. In addition, if the two markers on this plasmid are considered typical of nuclear markers that

Figure 6.1

Sensitivity to Chloramphenicol of Regenerating Protoplasts.

The PEG-calcium nitrate transformation procedure was performed on $6x10^6$ *N. tabacum* leaf mesophyll protoplasts as described in Materials and Methods, but DNA was not included. Aliquots of $1x10^6$ protoplasts were cultured in agarose beads in the presence of chloramphenicol at concentrations ranging from 0 to 50 µg/ml as indicated. The cultures were photographed after six weeks of culture.

Figure 6.1

confer chloramphenicol or kanamycin resistance, as seems the case at least for *nptII* (Velten and Schell 1985, and see below), a general conclusion may be drawn about the relative effectiveness of each combination of marker gene and antibiotic.

DNA of pCAP212 was digested at its unique *Hin* dIII site and 40 µg was included in a PEG-calcium nitrate transformation experiment as described in Materials and Methods. After embedding in agarose, the sample was divided into 8, and one portion cultured with each of 0, 5, 10, 20, 30, 40, and 50, µg chloramphenicol/ml, and one with 50 µg kanamycin/ml. After six weeks between 100 and 200 resistant calli were estimated amongst those selected with kanamycin. This selection system has frequently been shown to be effective in distinguishing transformants from non-transformants (for example Herrera-Estrella *et al.* 1983; Czernilofsky *et al.* 1986a), and all such resistant calli can be assumed to represent true transformants, though this was not ascertained directly. Growth of the calli cultured with chloramphenicol was again observed to be progressively reduced at higher antibiotic concentrations. However, significant differences in growth rate were not observed between calli treated at each particular chloramphenicol concentration, and potential transformants could not be discriminated. Eventually calli cultured with less than 30 µg chloramphenicol /ml outgrew the agarose bead and were discarded.

Those calli grown in chloramphenicol at 30 µg/ml or more failed to grow, so were cultured further in the absence of antibiotic to encourage development of transformants which may have been alive but unable to grow. After about two weeks, calli began to develop from the beads, with some growing noticeably faster than others, particularly amongst calli originally treated with chloramphenicol at 50 µg/ml. These were transferred from each sample to petri dishes containing solidified Murashige and Skoog (MS) medium supplemented with chloramphenicol at 40 µg/ml. Eventually the calli that remained in the agarose beads in the absence of selection overgrew the beads precluding identification of further putative transformants. This confirms previous observations that chloramphenicol does not immediately kill sensitive calli under minimal selection conditions (De Block *et al.* 1984).

Of 20 calli transferred from the sample selected at 50 µg/ml, four continued growth on the solidified MS medium supplemented with chloramphenicol. These four calli were assayed for CAT activity and all were positive (Figure 6.2) so were assumed to be transformants. None of the remaining calli including those from the samples selected with chloramphenicol at 30 or 40 µg/ml developed sufficiently to be assayed, suggesting that they were not transformed. In agreement with a previous report (De Block *et al.* 1984), increased discrimination between sensitive and resistant calli appeared to result during growth on solidified medium, and these preliminary observations were confirmed in a subsequent

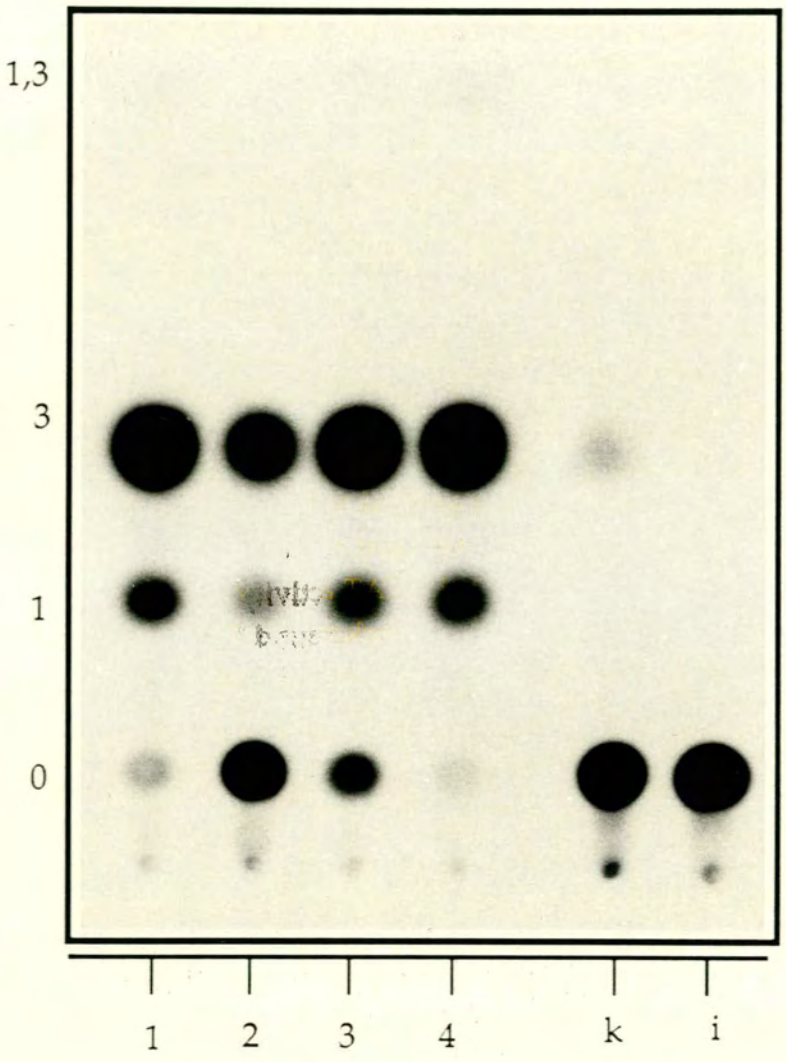
Figure 6.2

Chloramphenicol Acetyltransferase Activity in Chloramphenicol Resistant Calli Recovered from Transformed Protoplasts Selected with Chloramphenicol at 50 µg/ml.

N. tabacum leaf mesophyll protoplasts were transformed with 40 µg of a *Hin*dIII digest of pCAP212 (this plasmid is linearised by digestion with *Hin*dIII; Velten and Schell 1985) using the PEG-calcium nitrate transformation procedure. Protoplasts were regenerated in agarose beads in the presence of chloramphenicol at 50 µg/ml, which prevented growth of the microcalli. Chloramphenicol was removed allowing growth of the microcalli to resume, and the 20 calli that appeared to be growing most rapidly were transferred to Murashige and Skoog medium supplemented with agar at 0.8% and chloramphenicol at 40 µg/ml. Four calli continued to grow and were assayed for CAT activity (lanes 1 to 4). k shows the activity in a chloramphenicol resistant callus that was known to display weak CAT activity (Figure 6.8). i shows the CAT activity in a chloramphenicol sensitive callus that was known to display only background CAT activity (Figure 6.8).

0, 1, and 3 indicate the positions of chloramphenicol and its two monoacetylated forms respectively.

Figure 6.2



series of experiments (section 6.5).

From this experiment it was concluded that at chloramphenicol concentrations required to inhibit or impair growth of untransformed calli, transformants are not sufficiently resistant to allow significantly faster growth. Once the chloramphenicol is removed, most calli begin to grow but transformants do so more rapidly, such that if the selection has been stringent enough, they can be discriminated from the majority of the other calli. This procedure was inefficient; discrimination of transformants in the agarose bead was difficult, and of those calli that appeared to be transformed, only 25% sustained growth on solid medium containing chloramphenicol and expressed *cat*. The number of apparently transformed calli recovered by selection with chloramphenicol represented only a few percent of the number recovered with kanamycin.

It was decided to attempt to improve selection with chloramphenicol by varying the selection regime before fundamentally changing the tissue culture system.

6.3.2 Selection of Putative Transformants with a Regime of Decreasing Chloramphenicol Concentration.

The conclusion from the initial experiments was that little difference exists between the growth response to chloramphenicol of transformed and untransformed cells. What distinction there is lies in their rates of recovery from inhibition of growth by chloramphenicol. It was postulated that if chloramphenicol was gradually removed from cultures in which it had stopped callus growth, the transformed cells may resume growth earlier than the rest, providing time for the distinction between them to become exaggerated. If growth of the calli was initially inhibited by the lowest effective concentration of chloramphenicol, it would presumably allow selection of transformants with lower resistances. A similar conclusion was reached by Pietrzak *et al.* (1986), who reported the successful recovery of transformants using a regime of decreasing chloramphenicol concentration; however, they gave no details except that 40 µg/ml were initially applied for two weeks.

In order to investigate the effectiveness of such selection procedures, protoplasts were again prepared from SR1 tobacco plants and transformed with 40 µg of *Hin*dIII digested pCAP212 as described in Materials and Methods. After 7 days the protoplasts were set in agarose, and the bead divided into four. One quarter was selected with kanamycin at 50 µg/ml, three were left unselected for one week, and then chloramphenicol was added at 40 µg/ml to two of these. Subsequently the chloramphenicol concentration was reduced by 10 µg/ml, in

one case weekly, in the other fortnightly. The selection regimes of the other quarters were left unaltered; these regimes are summarised in the table below.

The calli cultured without chloramphenicol overgrew the bead. The quarter of the bead subjected to kanamycin selection yielded 50 to 100 transformants, which amounted to a transformation frequency of 0.01-0.1% which is typical of the PEG-calcium nitrate transformation technique (Wirtz *et al.* 1987). Seven weeks after protoplast isolation, when culture D contained chloramphenicol at 20 μg/ml and culture C lacked chloramphenicol, two apparently resistant calli were clearly discernible on each. Initially these calli were distinguished by their green colour, but within two weeks grew to become several times larger than the rest which were brown and pale. Following this, culture C became overgrown in the absence of chloramphenicol, with no further transformants being distinguished. In contrast three more potential transformants appeared in culture D as the chloramphenicol concentration was reduced to 10 μg/ml. This suggested that resistant calli appear asynchronously, and can be recovered so long as the untransformed calli remain sufficiently inhibited. To prolong this period for discrimination following removal of antibiotic from culture D, chloramphenicol was replaced in the medium at 10 μg/ml for one week each time the culture showed signs of general growth. In this way, the growth of untransformed calli was suppressed for several weeks before the culture died, and an additional seven resistant calli were recovered. No such calli resulted when DNA was omitted from the transformation.

All chloramphenicol resistant calli so obtained were transferred to solidified MS medium containing chloramphenicol at 10 μg/ml. All continued growth, although one that had barely begun to grow in the bead was slow to initiate growth on the plate.

At the later stages of selection, several calli had appeared which were green, but diffuse and surrounded by colourless tissue from which separation was difficult. To evaluate whether all or part of such calli was transformed, one was dissected into its various components and each grown on MS medium without chloramphenicol until large enough for a CAT assay. To show that CAT activity was not generally induced in the calli by the culture conditions but was linked to the resistant phenotype, several of the small, brown, chloramphenicol sensitive calli were similarly transferred to MS medium not supplemented with chloramphenicol. On this medium they initiated growth and were assayed for CAT together with the potential transformants described above.

All chloramphenicol resistant calli contained high levels of CAT activity, whereas the brown chloramphenicol sensitive calli did not (Figure 6.3). Analysis of the various sectors of the callus with mixed morphology showed that tissue derived from sectors which had been green expressed CAT, but tissue derived from only two of the three white sectors did so, suggesting that such white sectors

Selection Regime Using Decreasing Chloramphenicol Concentrations.

0. Week		Transformation ⇓		
1.		Beading, Division and Selection		
	A.	B.	C.	D.
	No selection	**Km**	**CAP**	**CAP**
	– ⇓	50 µg/ml ⇓	0 µg/ml ⇓	0 µg/ml ⇓
2.	Continued	Continued	40 µg /ml ⇓	40 µg /ml ⇓
3.			30 µg/ml ⇓	40 µg/ml ⇓
4.			20 µg/ml ⇓	30 µg/ml ⇓
5.			10 µg/ml ⇓	30 µg/ml ⇓
6.			0 µg/ml ⇓	20 µg/ml ⇓
7.			0 µg/ml ⇓	20 µg/ml ⇓
8.			0 µg/ml ⇓	10 µg/ml ⇓
9.			0 µg/ml ⇓	10 µg/ml ⇓
10.			0 µg/ml	0 µg/ml ⇓
				10µg/ml As Required

After transformation and beading, the protoplasts were divided into four portions (A to D) and cultured under the selection conditions shown. A was left unselected. B was selected with Kanamycin at 50 µg/ml. C and D were left for a week without antibiotic and chloramphenicol was then added at 40 µg/ml; subsequently the chloramphenicol concentration was reduced by 10 µg/ml at weekly (C) or fortnightly (D) intervals. After growth of culture D without antibiotic, Chloramphenicol was replaced as required to suppress general growth of the culture and allow transformants to emerge.

Figure 6.3

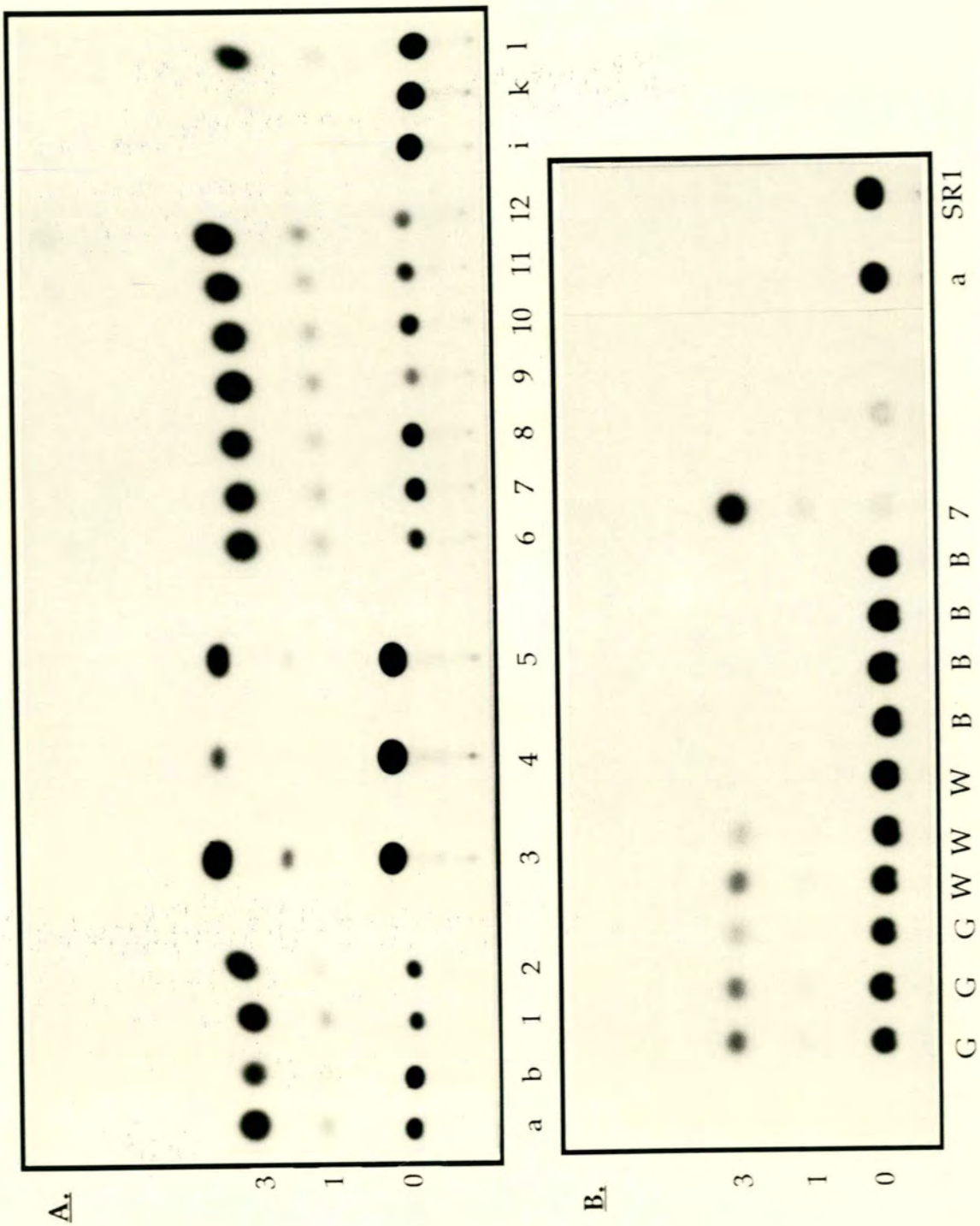
Chloramphenicol Acetyltransferase Activity in Chloramphenicol Resistant Calli Recovered from Transformed Protoplasts Regenerated in the Presence of Decreasing Chloramphenicol Concentrations.

A. $1x10^6$ *N. tabacum* leaf mesophyll protoplasts were transformed with 40 µg of a *Hin* dIII digest of pCAP212, and cultured in agarose beads in four aliquots of $2.5x10^5$ protoplasts. Chloramphenicol was added to two of the cultures at a concentration of 40 µg/ml two weeks after transformation. The chloramphenicol concentration was decreased by 10 µg/ml in one case at weekly intervals, resulting in the recovery of two potential transformants (a and b), and in the other case at fortnightly intervals, resulting in the recovery of twelve potential transformants (1 to 12) during several weeks as described in the text. The CAT activity in callus tissue from each potential transformant was assayed and is shown in this figure. Samples labelled i, k, and l show the CAT activity in control calli; The first of these demonstrates only background CAT activity, and the other two contain different levels of CAT activity due to expression of integrated pCAP212 sequences (Figure 6.8).

B. In the experiment above, several diffuse calli containing pale green (G) and white (W) sectors were observed. Their phenotype was intermediate between those of the apparently resistant and sensitive calli. One such callus was dissected into its various components which were grown on Murashige and Skoog medium without supplementation with chloramphenicol until large enough to be assayed for CAT activity. In addition, four of the brown chloramphenicol sensitive calli (B) were similarly cultured on Murashige and Skoog medium and assayed for chloramphenicol activity. The activity in each of these was compared to that in callus 7 whose activity is shown in **A**.

This figure also shows the CAT activity in a callus that was derived from untransformed protoplasts and appeared to be chloramphenicol resistant during selection in the agarose bead (a). SR1 shows the CAT activity in untransformed *N. tabacum* tissue.

Figure 6.3



may be chimaeric. It seems that in these selection conditions, development of a green colour, and presumably chloroplasts, combined with rapid growth of the callus is a reliable indicator of transformation. Various alternative regimes for reducing chloramphenicol concentration from 40 μg/ml to zero have been followed and assessed, but little difference in recovery of transformants was detected amongst them. Finally one regime was adopted in which calli were cultured for two week periods with chloramphenicol at concentrations of 40 μg/ml, then 10 μg/ml, then zero, and subsequently at 10 μg/ml as necessary. This selection procedure is referred to in future as the standard selection procedure.

Following PEG-calcium nitrate transformation experiments on several occasions over two years, the standard selection procedure reliably yielded resistant calli, but a false positive was not selected (for example Figure 6.4). Further evidence that these calli were true transformants was obtained by assaying for expression of the nonselected NPTII gene of pCAP212 (Figure 6.9 A), and by Southern blotting using a DNA probe containing CAT gene sequence (Figure 6.5). In controls where protoplasts were not put through the transformation process, or where transforming DNA was omitted or did not expresses *cat*, no resistant calli emerged. The results of these experiments are summarised in Table 6.1. In one experiment not included in this table an apparently resistant callus was observed from untransformed protoplasts. This however failed to grow on MS with 10 μg/ml chloramphenicol, and showed no CAT activity (Figure 6.3B).

The number of calli apparently transformed with pCAP212 that were recovered using the chloramphenicol selection procedure was about 10 to 20% of the number obtained when kanamycin was used to select transformants from the same population. Despite the large difference in the mean recovery of transformants using each antibiotic, the significance of this difference in a t-test is between 90% and 95% owing to the large variance of the data. However, it appears that during selection fewer initial transformants may express sufficient activity from *cat* than from *nptII* to confer resistance to the respective antibiotics. Variation in the level of expression of transforming DNA integrated into the genomes of independent transformants has frequently been documented (for example Czernilofsky *et al.* 1986a and b, Dean *et al.* 1988, Weising *et al.* 1988). The greater efficiency with which kanamycin resistant calli are recovered appears not to result from the kanamycin resistance marker on pCAP212 being unusually effective because pLGV1103 gave similar transformation frequencies. Similarly, there is no evidence that the chloramphenicol resistance marker of pCAP212 is particularly ineffective because transformation of protoplasts with plasmids pRT-T1 and pRTpreβcat, which also carried *cat*, yielded fewer transformants;

Table 6.1
Recovery of Resistant Calli after Selection with Kanamycin and Chloramphenicol.

Vector	Selectable Marker		Anti-biotic	Experiment Number 1	2	3	4	5	6	Av.[b.]
pCAP212	*nptII*	*cat*	Km	60	44	194	260	28	106	115
pLGV1103	*nptII*	–	Km	–	96	–	196	8	–	100
pCAP212	*nptII*	*cat*	Cm	24	10	12	38	0	22	18
pRT-T1	–	*cat*	Cm	10	3	16	7	3	23	10
pRTpreβcat	–	*cat*	Cm	–	3	0	6	0	2	2
pLGV1103	*nptII*	–	Cm	–	0	–	0	0	–	0
a.	–	–	Cm	0	–	0	–	–	–	0
a.	–	–	Km	–	–	0	–	–	–	0

Columns headed 1-6 show the results of six independent transformation experiments. Protoplasts were transformed by the PEG-calcium nitrate technique and selected using the standard procedure for chloramphenicol and kanamycin (Materials and Methods). Each experiment included several of the *cat* and *nptII* containing plasmids indicated on the left; dashes indicate that a particular plasmid was not included. The number of transformed calli recovered was determined 11 weeks after transformation; no additional transformants were recovered thereafter. Either $5x10^5$ or $1x10^6$ protoplasts were cultured in each sample, and the number of resistant calli recovered is expressed in each case as the number recovered per million cultured protoplasts. The top two rows show results with plasmids containing kanamycin resistance markers, the second three, those with chloramphenicol resistance markers, and the third three those with the negative controls.

a. In these samples, either vectors with no selectable marker were used, or protoplasts were untransformed.

b. Mean recovery per 10^6 protoplasts transformed.

The phenotype of all chloramphenicol resistant calli was confirmed by continued growth on MS supplemented with chloramphenicol at 10 μg/ml as in Materials and Methods.

Figure 6.4

Selection for Transformants Amongst Regenerating Protoplasts Using Chloramphenicol and Kanamycin.

A. This photograph shows an example of protoplasts regenerating in the agarose bead culture system used for selection of transformed calli. Two samples of $1x10^6$ *N. tabacum* mesophyll protoplasts were either transformed with pCAP212 using the PEG-calcium nitrate transformation procedure and cultured in the presence of kanamycin (Km), or were left untransformed and cultured in the presence of either kanamycin or chloramphenicol (Cm), all as described in Materials and Methods. pCAP212 can confer a kanamycin resistant phenotype to transformed plant tissue owing to expression of the NPTII gene of Tn*5* from the T-DNA 2' promoter (Velten and Schell 1985). These samples are taken from experiment number 3 in Table 6.1, and there are $2.5x10^5$ protoplasts on each dish.

B. This photograph shows the samples transformed with pCAP212 or pLGV1103 derived from experiment 2 of Table 6.1. Plasmid pLGV1103 confers a kanamycin resistant phenotype to transformed plant cells owing to expression of the NPTII gene of Tn*5* from the nopaline synthase promoter and octopine synthase polyadenylation signal (Czernilofsky *et al.* 1986a). Plasmid pCAP212 confers kanamycin resistance to transformed plant cells as described above, and also chloramphenicol resistance owing to expression of the Type I CAT gene from the T-DNA 1' promoter (Velten and Schell 1985). Each sample contains $2.5x10^5$ *N. tabacum* mesophyll protoplasts transformed using the PEG-calcium nitrate transformation procedure and cultured with kanamycin (Km) or chloramphenicol (Cm) as described in Materials and Methods. Cultures were photographed nine weeks after transformation. Two chloramphenicol resistant calli and thirteen kanamycin resistant calli transformed with pCAP212, and twelve kanamycin resistant calli transformed with pLGV1103 are discernible on the photograph.

Figure 6.4

A.


Untransformed: Km
Untransformed: Cm
pCAP212: Km

B.


pCAP212: Km
pCAP212: Cm
pLGV1103: Km
pLGV1103: Cm

Figure 6.5

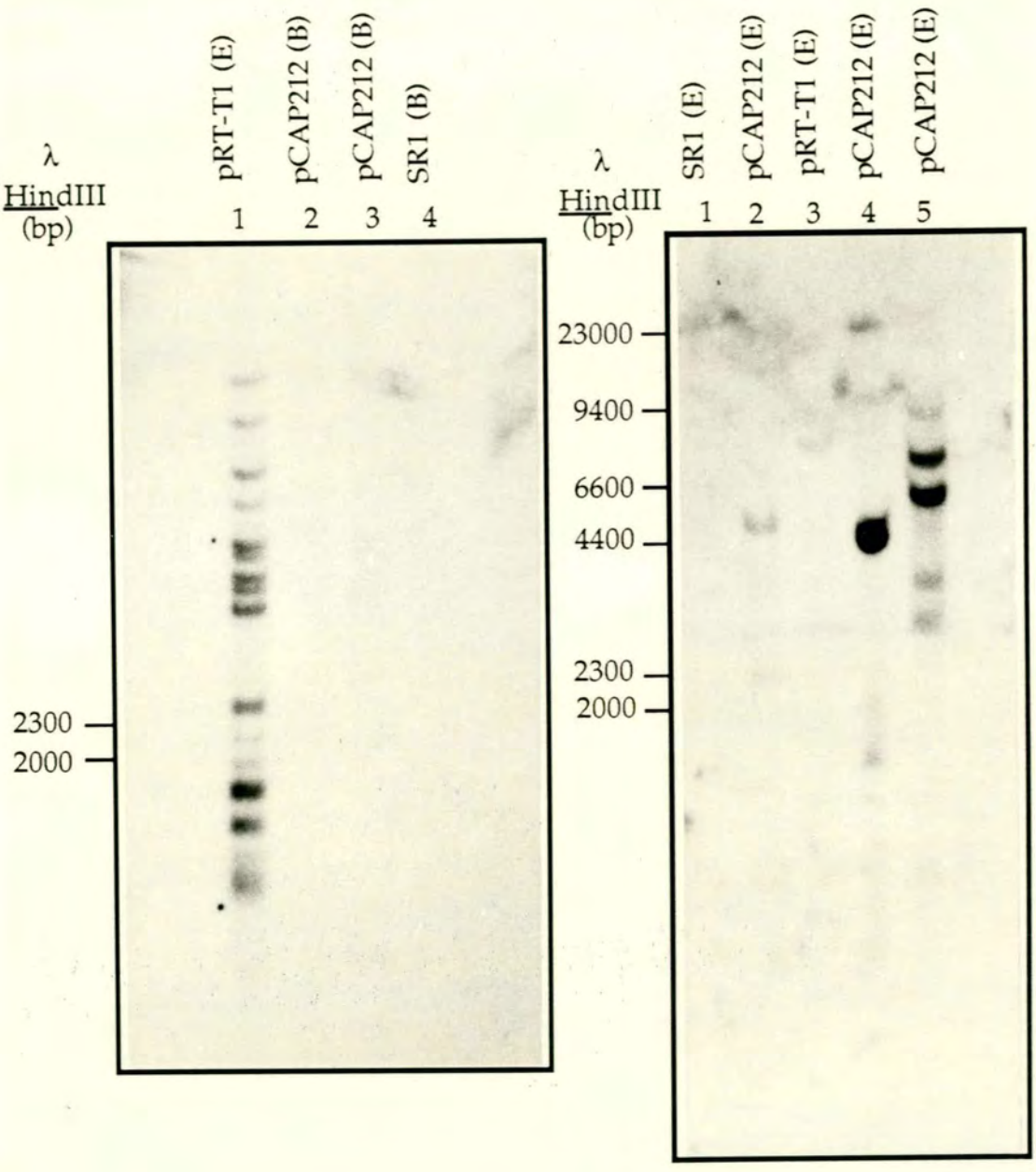
Southern Blot Hybridisation Analysis of Genomic DNA Isolated from Chloramphenicol Resistant Calli.

Approximately 10 µg of genomic DNA isolated from several kanamycin or chloramphenicol resistant calli was digested with either *Eco*RI (E) or *Bam*HI (B). Digestion products were separated by agarose gel electrophoresis and the DNA was blotted onto Hybond-N nylon membranes (Amersham International plc) using an LKB 2016 Vacugene vacuum blotting unit according to the manufacturers recommendations. Filters were illuminated with U.V. light for between 2.5 and 4.5 minutes and baked under vacuum at 80^0C for 60 minutes. Prehybridisation was at 65^0C for 16 hours in 6xSSC, 5x Denhardts Solution, 0.5% SDS and 200 µg/ml herring sperm DNA (see Materials and Methods). The 850 bp *Eco*RI to *Xba*I fragment of pRT-T1 that contains the Type I CAT gene (Figure 6.6 B) was used to generate the probe using random oligonucleotide primers as described in Materials and Methods. Hybridisation was at 65^0C for 16 hours in 5xSSC, 5x Denhardts Solution, 0.5% SDS, 10 mM EDTA pH 8.0, 250 µg/ml herring sperm DNA and 10% dextran sulphate. Filters were washed and exposed to X-ray film as described in Materials and Methods. The autoradiograph was photographed, and the top of the photograph corresponds to the position of the wells of the agarose gel.

A. Hybridisation to genomic DNA isolated from a chloramphenicol resistant (lane 1) and two kanamycin resistant (lanes 2 and 3) calli transformed with pCAP212, and also from untransformed *N. tabacum* cv. Petit Havana SR1 leaves. Strong hybridisation was observed to several bands in lane 1 and weaker hybridisation was observed in lanes 2 and 3. DNA from untransformed tissue did not hybridise with the probe.

B. Hybridisation to genomic DNA isolated from untransformed *N. tabacum* cv. Petit Havana SR1 leaves (lane 1), chloramphenicol resistant calli recovered from transformation with pCAP212 (lanes 2 and 4) and pRT-T1 (lane 3) and kanamycin resistant calli recovered from transformation with pCAP212 (lane 5). Genomic DNA from untransformed tissue showed no hybridisation to the probe, but hybridisation was observed to the transformants in lanes 2, 3 and 5. Hybridisation was observed in lane 4 but its significance was less clear.

Figure 6.5


λ
HindIII
(bp)
pRT-T1 (E)
pCAP212 (B)
pCAP212 (B)
SR1 (B)
1
2
3
4
2300
2000
λ
HindIII
(bp)
SR1 (E)
pCAP212 (E)
pRT-T1 (E)
pCAP212 (E)
pCAP212 (E)
1
2
3
4
5
23000
9400
6600
4400
2300
2000

however there was no internal control such as *nptII* in the latter plasmids to exclude the possibility that they simply transformed plant cells less frequently than pCAP212 rather than confered resistance to fewer transformed calli.

It was concluded that this chloramphenicol based selection procedure was reliable, repeatable, and a significant improvement on those previously reported. The frequency of recovery of chloramphenicol resistant calli is 10-20% of that for kanamycin resistance. For most experimental purposes this frequency is adequate, so plasmids expressing *cat* can be routinely used in combination with chloramphenicol to generate and select nuclear transformants. Whether this selection strategy will be sufficient to recover mitochondrial transformants is discussed later, but it does have one advantage for mitochondrial transformation in particular. That is, following transformation, the selectable gene will be present probably at only one copy per cell; amplification may not have occurred when selection is applied so it is important to know that such cells could survive. I have shown that with chloramphenicol this can be assumed as even untransformed cells without a CAT gene can survive; this is in contrast to the other more stringent selective agent, kanamycin, which I have found to be lethal to sensitive cells. Furthermore, the delay after transformation in applying selection, and the long subsequent culture period with intermittent selection would favour the segregation of resistant cell lines from slowly growing calli.

6.4 A Variant of CAT that is Targeted to the Mitochondria Allows Selection of Chloramphenicol Resistant Transformants.

The experiments described above were performed with vectors that are designed to facilitate the recovery of nuclear transformants. CAT is therefore most likely to be located in the cytosol, although Bunker and Moore (1988) have proposed that in mammalian cells it can be secreted into the medium. The questions remain whether CAT will retain activity in the mitochondrion, and whether this will allow selection of resistant cells. Boutry *et al.* (1987) fused *cat* to a sequence encoding the transit peptide of the β subunit of the mitochondrial F1 ATP synthase from tobacco. The transit peptide normally directs this subunit to the mitochondrion from its site of synthesis in the cytosol, and was shown to do the same for the CAT fusion polypeptide. Immunological and biochemical assays of tobacco tissue transformed with the fused gene showed that all detectable CAT protein and activity was localised in the mitochondria. Such tissue therefore expresses CAT activity, but no attempt was made to assess its resistance to chloramphenicol. Plasmid pDS5-β encoding the CAT derivative used above was provided by Dr. M. Boutry in order that such an analysis could be made. It must be

noted that this experiment relied on the assumption that in any transformants that were recovered the majority of the CAT activity contributing to their resistant phenotype was located in the mitochondria; even though Boutry *et al.* (1987) have shown that in cells transformed with the chimaeric CAT gene the mitochondria contain all detectable CAT activity, the possible contribution of a residual amount of cytosolic CAT can not be excluded. None-the-less, it was of interest to discover what would be the effect, if any, of localising the majority of CAT activity in the mitochondria even though the observations would be difficult to interpret conclusively.

The protein fusion studied by Boutry *et al.* (1987) was termed preβcat, and the open reading frame encoding the protein was termed *preβcat* . Plasmid pDS5-β is designed for expression of *preβcat* in prokaryotic systems, so to allow its expression in plant cells it was transferred into the polylinker of pRT101 which is a plant nuclear expression vector containing a promoter and polyadenylation signal derived from Cauliflower Mosaic Virus (Figures 4.8 and 6.6A). In pDS5-β, *prebcat* is flanked by unique *Eco*RI and *Xba*I recognition sites at the 5' and 3' end respectively, and recognition sites for these enzymes also occur between the 35S promoter and polyadenylation site of pRT101. Digests with these enzymes of pDS5-β and pRTCAT100 (a derivative of pRT101, Figures 4.8 and 4.9 A) were mixed, ligated, and transformed into HB101. Plasmid DNA from 12 ampicillin resistant clones was digested with *Hin*dIII and one containing the DNA fragment encoding preβcat at the *Eco*RI and *Xba*I sites of pRT101 was identified. The structure of this plasmid was confirmed by digestion with *Pst*I, *Hin*dIII, *Xba*I with *Eco*RI and *Eco*RI with *Pst*I (Figure 6.6B) and the plasmid was called pRTpreβcat. This plasmid will direct expression of a protein identical to that studied by Boutry *et. al.* (1987).

As a control, a similar plasmid that expresses CAT in the cytosol was constructed. The sequence encoding the transit peptide of preβcat is located on a 280 bp *Hin* dIII fragment. This was removed from pDS5-β by digestion with *Hin*dIII and religating the vector (Figure 6.6A). Ampicillin resistant *E. coli* colonies resulting from transformation with the ligation products were screened for plasmids that lacked the 280 bp *Hin*dIII fragment by digesting plasmid DNA with *Hin*dIII and *Hin*dIII with *Xba* I (not shown). The reading frame newly generated in this plasmid encodes a wild type CAT enzyme (Boutry *et al.* 1987) and.was transferred into the pRT101 polylinker as above (Figure 6.6A and B) to produce the plasmid pRT-T1.

Figure 6.6A

Construction of pRT-T1 and pRTpreβcat.

The relevant region of pDS5-β is shown in Part 1 (for simplicity, in all cases the vector sequences containing the β-lactamase gene and origin of replication have been omitted from the drawings of the plasmids). It contains the Type I CAT gene from Tn*9* (Hatched Box) fused by its N-terminus to the transit peptide of the F1-F0 ATP synthase β-subunit precursor (Stippled Box). The entire fused coding sequence was removed on a fragment of about 1.2 kb generated by digestion with *Eco*RI (E) and *Xba*I (Xb). This fragment was inserted between the *Eco* RI and *Xba* I sites of pRTCAT100 shown in Part 3. This latter plasmid contains the Type I CAT gene from *P. mirabilis* (Narrow Hatching) inserted in the polylinker of pRT101, a plant nuclear expression vector that contains the 35S promoter (35S) and a polyadenylation signal (pA) from Cauliflower Mosaic Virus (see Figure 4.8). Plasmid pRTCAT100 contains *Eco*RI recognition sites within the coding sequence of *cat* and at its 5' end, and contains an *Xba*I site at the 3' end of *cat*. Thus the *P. mirabilis* CAT gene can be removed from pRTCAT100 with *Eco*RI and *Xba*I, and replaced by the 1.2 kb fragment encoding preβcat. A plasmid containing this fragment was called pRTpreβcat (Part 4), and restriction digests are shown in Figure 6.6B.

As a control, a plasmid similar to pRTpreβcat but lacking the preβ transit sequence was constructed. To do this, the transit sequence was removed from pDS5-β by digestion at the *Hin*dIII sites, followed by religation of the vector to produce the plasmid represented in Part 2. The CAT gene in this plasmid retains its authentic initiation codon, and can thus be expressed to produce a wild-type enzyme. The modified *Eco*RI to *Xba*I fragment from this plasmid was transferred into pRTCAT100 exactly as described for construction of pRTpreβcat, and the resulting plasmid was called pRT-T1 (Part 4).

P, Sp, and B, indicate recognition sites for the restriction endonucleases *Pst*I, *Sph*I and *Bam*HI respectively.

Figure 6.6 A

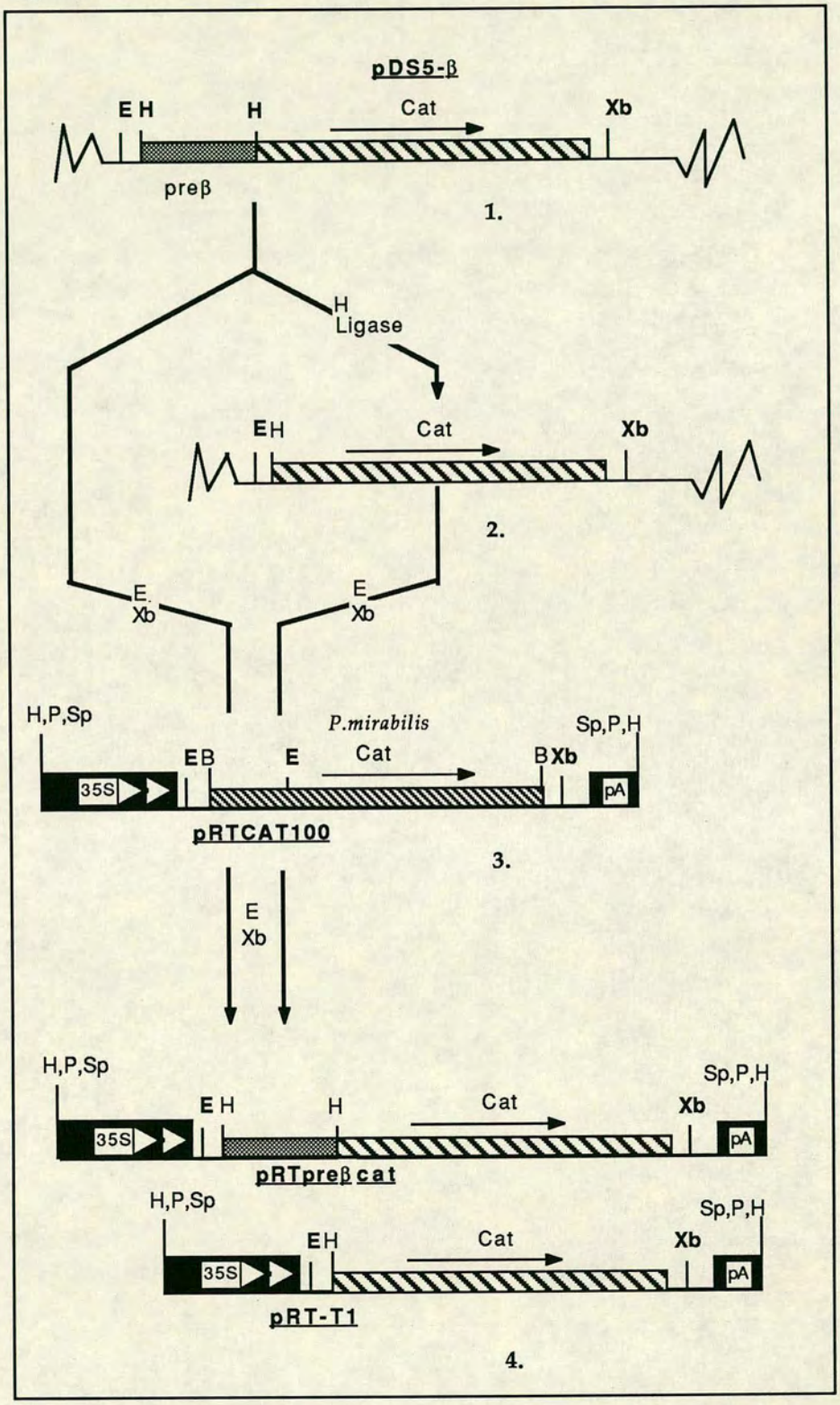

pDS5-β
E H
H
Cat
Xb
preβ
1.
H
Ligase
EH
Cat
Xb
2.
E
Xb
E
Xb
H,P,Sp
P.mirabilis
Sp,P,H
EB
E
Cat
BXb
35S
pA
pRTCAT100
3.
E
Xb
H,P,Sp
Sp,P,H
E H
H
Cat
Xb
35S
pA
pRTpreβcat
H,P,Sp
Sp,P,H
EH
Cat
Xb
35S
pA
pRT-T1
4.

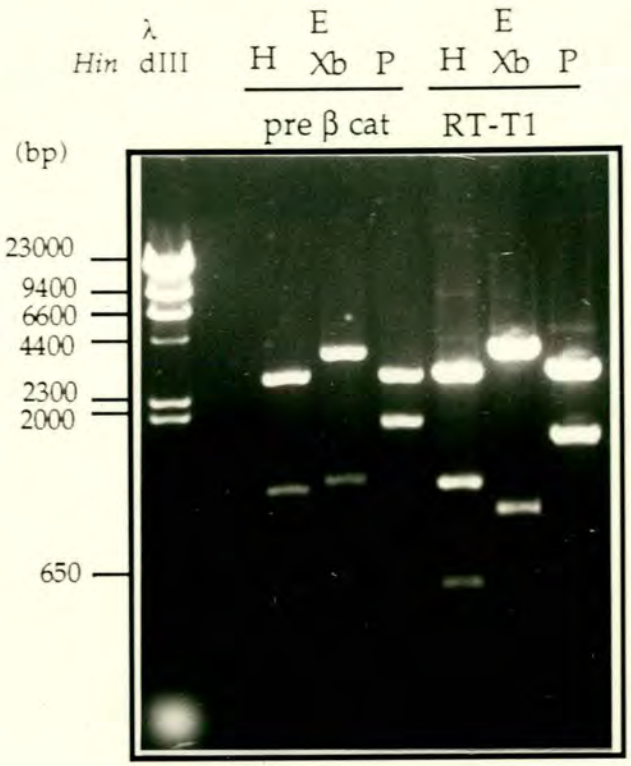
Figure 6.6 B

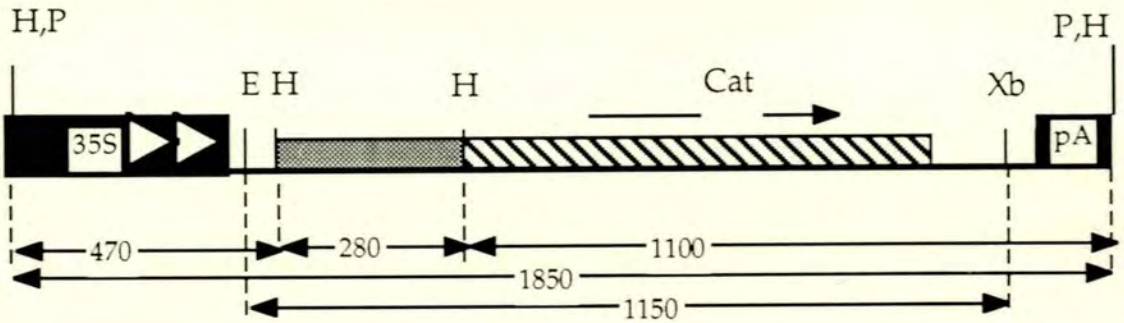
Restriction Endonuclease Digestion Analysis of pRTpreβcat and pRT-T1.

Plasmids pRTpreβcat and pRT-T1 were digested with the restriction endonucleases *Eco*RI (E), *Hin*dIII (H), *Pst*I (P) or *Xba*I (Xb), digestion products were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed under U.V. illumination. A *Hin*dIII digest of bacteriophage λ DNA was used as a DNA size marker, and the approximate size of each band in base pairs is shown.

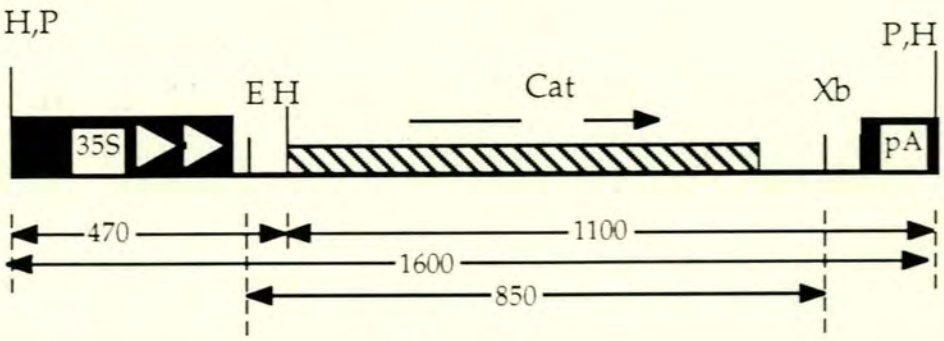
The origin and approximate size in base pairs of the fragments expected in each digest are shown in the diagrams below the gels. These are drawn similarly to the equivalent diagrams in Figure 6.6 A.

Figure 6.6 B





pRTpre β cat



pRT-T1

Before stable transformation was attempted, transient expression experiments were performed to ensure that both pRT-T1 and pRTpreβcat expressed CAT activity. This analysis showed that both plasmids expressed similarly high levels of CAT activity (Figure 4.11D). Boutry *et al.* (1987) were unable to detect unprocessed forms of preβcat in stably transformed tobacco tissue and all intracellular CAT appeared to be located in the mitochondria. In the transient expression assays reported here, the CAT activity in the protoplasts was analysed 3 days after DNA transfer, however Pröls *et al.* (1988) found that synthesis of CAT in transient expression assays is complete between four and seven hours after DNA transfer; it is assumed that the CAT activity measured in transient expression analysis of pRTpreβcat was located in the mitochondria at the time the assay was performed.

Having shown that both pRT-T1 and pRTpreβcat can express CAT in protoplasts, they were used in attempts to generate stable chloramphenicol resistant transformants using the PEG-calcium nitrate transformation technique. For this purpose DNA was digested with either *PstI* or *BglI* to generate linear molecules before transformation. Transformation frequencies obtained with these plasmids were compared with each other and also to those obtained with pCAP212 in parallel transformations. The results of these experiments are compiled in Table 6.1.

Encouragingly, resistant calli that express CAT activity were obtained with both plasmids (Figure 6.7). Slightly fewer were recovered with pRT-T1 than pCAP212, but in a t-test the difference was not significant because the data are highly variable, probably due to differences between samples in protoplast survival and in longevity of the cultures. A more significant difference (90% to 95%) was observed between pRT-T1 and pRTpreβcat. One explanation for the lower transformation efficiency of the latter may be that CAT confers resistance to chloramphenicol less effectively in the mitochondrion than the cytosol, thus fewer calli may express sufficient activity to survive the selection process. The implication is that if CAT is synthesised directly in the mitochondria following mitochondrial transformation, weakly resistant calli may result.

As no internal control for transformation, such as a second selectable marker, was included, it remains possible that the difference in the efficiency with which transformants are recovered using the cytosolic and mitochondrial forms of CAT may not be due entirely to a difference in the resistance they confer. It may be for example that the two plasmids are equally effective in conferring resistance to chloramphenicol, but that for some reason pRTpreβcat generated fewer initial transformants, or that some independent effect associated with preβcat adversely affects the development of cells expressing *preβcat* . For example, one possibility is that the import of preβcat into mitochondria stresses plant cells, and reduces

Figure 6.7

Chloramphenicol Acetyltransferase Activity in Plants and Calli Transformed with pRTpreβcat, pRT-T1 and their Derivatives, and Recovered by Selection for Chloramphenicol Resistance.

A. The CAT activity demonstrated by several chloramphenicol resistant transformants recovered using several different transformation techniques are shown. These include the following :

i. Three chloramphenicol resistant calli recovered from PEG-calcium nitrate transformation of 10^6 *N. tabacum* cv. Petit Havana SR1 protoplasts using 40 μg of *Bgl* I digested pRT-T1 (T1). These calli were obtained from experiment number 5 in Table 6.1.

ii. Two calli obtained by cocultivation of *N. tabacum* cv. Petit Havana SR1 protoplasts with *A. tumefaciens* strain GV3101 MP90RK harbouring pPCRTβcat as described in Chapter 7 (section 7.1.3.2 and Figure 7.1). untr shows the CAT activity in untransformed *N. tabacum* cv. Petit Havana SR1 callus tissue.

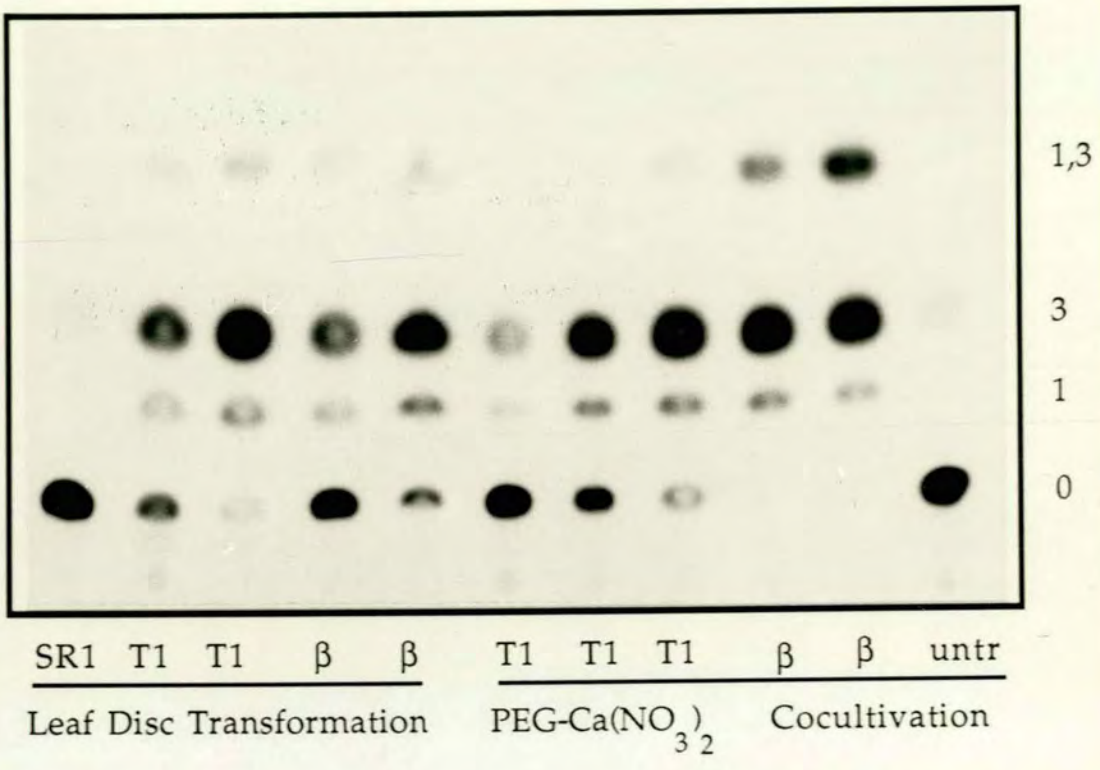
iii. Leaf tissue from four chloramphenicol resistant shoots derived from transformation of *N. tabacum* cv. Petit Havana SR1 leaf discs with *A. tumefaciens* strain GV3101 MP90RK harbouring pPCRTβcat (β) or pPCRT-T1 (T1) as described in Chapter 7 (section 7.1.3.1 and Figure 7.1). Shoots from all four plants were able to form roots in the presence of 10 μg/ml chloramphenicol. SRI shows the CAT activity in leaf tissue from an untransformed *N. tabacum* cv. Petit Havana SR1 plant.

B. The CAT activity demonstrated by three chloramphenicol resistant calli recovered from experiment number 2 in Table 6.1 in which 10^6 *N. tabacum* cv. Petit Havana SR1 protoplasts were transformed with 40 μg of pRTpreβcat (β) that had been digested with *Pst* I. 1103 shows the CAT activity in a kanamycin resistant transformant recovered from the same experiment using 40μg of pLGV1103 that had been digested with *Eco* RI. pLGV1103 does not contain *cat* .

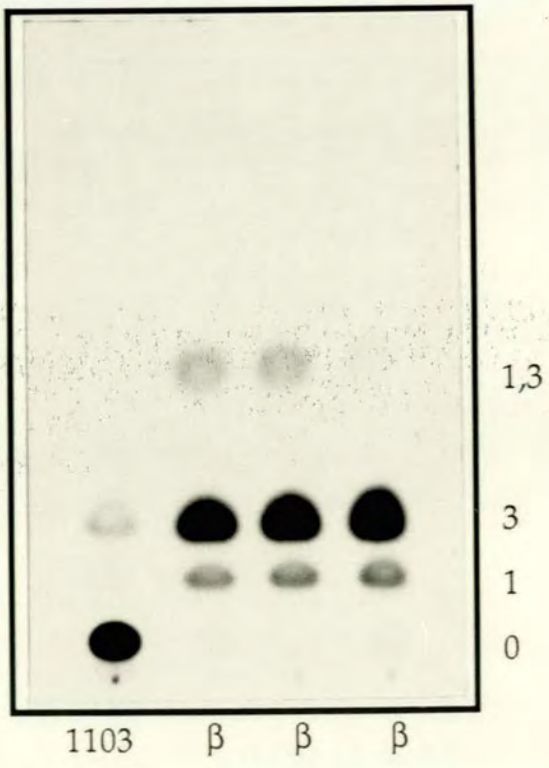
0, 1, 3, and 1,3; the positions of chloramphenicol, its two monoacetylated derivatives and its diacetylated derivative respectively.

Figure 6.7

A.


1,3
3
1
0
SR1 T1 T1 β β T1 T1 T1 β β untr
Leaf Disc Transformation
PEG-Ca(NO3)2 Cocultivation

B.


1,3
3
1
0
1103 β β β

their viability, particularly if its import is inefficient. The CAT genes from pRT-T1 and pRTpreβcat have been transferred into Ti plasmid derivatives that also carry *nptII* markers to act as internal controls. In preliminary experiments with these plasmids, the pRTpreβcat derivative generated slightly fewer kanamycin resistant transformants as well as fewer chloramphenicol resistant ones (Chapter 7). Whilst consistent with the possibility that preβcat exerts some adverse effect or that in the experiments of Table 6.1 pRTpreβcat generated fewer initial transformants, these preliminary results suggest that such effects can not account entirely for the lower efficiency of recovery of transformants using pRTpreβcat.

Of greater concern is that the absolute frequency with which resistant calli were obtained after transformation with pRTpreβcat was only about 2 per 10^6 protoplasts transformed. Again, as no internal control for transformation was included, the possibilities remain that for some reason pRTpreβcat transformed plant cells inefficiently, or that preβcat adversely affected transformants, although these explanations are questionable as discussed above. The third possibility is that the transformation experiments were performed inefficiently. However, the average transformation frequency obtained in these experiments when selecting for *nptII* of pCAP212 was 10^{-4} which is within the range normally obtained with the PEG-calcium nitrate procedure; furthermore, of the three plasmids tested, the CAT gene in pCAP212 allowed the recovery of chloramphenicol resistant calli with the greatest efficiency, but still was only ten fold more efficient than pRTpreβcat. Thus even if it is supposed that preβcat does in fact confer chloramphenicol resistance as effectively as its cytosolic counterparts, the maximal efficiency with which transformants would be expected to be recovered is about 20 per million protoplasts. In the best cases, transformation of yeast with a mitochondrial marker has been reported to be about 2,000 times less frequent than with a nuclear marker (Johnston *et al.* 1988, Fox *et al.* 1988b). If this differential also applies to plant cells, in order to recover each mitochondrial transformant it will be necessary to transform a number of cells equivalent to that required to recover about 2,000 nuclear transformants. To recover two thousand chloramphenicol resistant calli would require, in the best case, a hundred transformations such as those described above, but a thousand if pRTpreβcat was used. This is clearly far from ideal. As selection for *nptII* of pCAP212 using kanamycin gave at least a five fold higher recovery of transformants than selection for *cat* , there may be many transformants available for recovery that are not surviving the selection procedure; clearly, selection of transformants with chloramphenicol could be further improved, but even then the transformation may have to be scaled up or the basic efficiency of transformation improved. This is discussed in Chapters 7 and 8.

It seems therefore that the resistance conferred by CAT in the mitochondria may be weaker than if it were cytosolic. Discussions in preceding chapters

concerning the suitability of various selective agents, selectable marker genes, and gene expression signals for use in mitochondrial transformation experiments have clearly demonstrated the difficulties presented by the lack of positive controls for most aspects of this mitochondrial transformation strategy. Support for the experimental strategy that was adopted comes from the observation that pRTpreβcat can be used, albeit less efficiently than other plasmids, to recover chloramphenicol resistant nuclear transformants. This observation however does not provide a positive control for selection of mitochondrial transformants. Recovery of mitochondrial transformants but not nuclear transformants requires segregation and probably amplification of the transforming DNA; additionally, mitochondrial transformants and nuclear transformants obtained with pRTpreβcat will be similarly resistant only if they express a similar level of CAT activity, something which cannot be assumed, and in pRTpreβcat transformants it is not possible to exclude the potential contribution to the resistant phenotype that may result from a small, undetected, pool of preβcat in the cytosol.

6.5 Establishment of a Rapid Procedure to Screen Potentially Transformed Calli for Resistance to Chloramphenicol.

Selection procedures are not completely effective at excluding non-transformants, 'escapes', from the population of apparent transformants. Thus following selection of transformants, it is usually necessary to provide supporting evidence that any particular individual is truly transformed. Following relatively effective selection procedures, such as those used above to recover chloramphenicol and kanamycin resistant nuclear tobacco transformants, almost all individuals examined will prove to be transformants, so relatively time consuming biochemical or genetic analyses can be performed on a few individuals to discover genuine transformants. Even in cases where genuine transformants represent no more than about 10% of the initially selected population, such individual 'screening' procedures are feasible; however, if the population contains transformants at an incidence of about 1% or less such procedures are not feasible for routine screening.

In this work, there is no positive control to show what degree of resistance to chloramphenicol a mitochondrial transformant would achieve, and it is thus difficult to decide what to look for when selecting potential transformants with chloramphenicol. As mitochondrial transformants may be weakly resistant to chloramphenicol, a large number of marginally resistant calli may have to be considered as potential transformants and then analysed. Most of the calli so selected are likely to be untransformed, so it was felt that a rapid and convenient

way of screening large numbers of potential transformants for nontransformed escapes would be useful. Southern blot hybridisation analysis of genomic DNA, and the CAT assay are techniques routinely used in the more reliable nuclear transformation experiments, but are too expensive and labour intensive for the screening procedure required in this work. Potentially transformed calli are routinely transferred to solid media and allowed to grow before complex analysis is undertaken. A simple and perhaps indirect screen for genuine transformation at this stage, would, if sufficiently sensitive, reduce the number of potential transformants to be analysed by the more direct techniques generally used. Screening for continued expression of the chloramphenicol resistant phenotype during this phase represented the most convenient of the potential alternatives. As described below, this was found to be a highly sensitive screen for expression of *cat* in nuclear transformants in agreement with the results of De Block *et al.* (1984) who reported the successful use of such a screen.

Calli generated in the cotransformation experiment of section 4.4.1 were used to evaluate the suitability of this potential screening procedure. Following transformation with a mixture of pCAP212, that expressed only *cat* , and pLGV1103, that expressed only *nptII*, kanamycin resistant calli had been recovered. The vast majority of these calli contain and express *nptII* of pLGV1103 to confer the kanamycin resistant phenotype; some will express only *nptII*, however some will also carry pCAP212 and express *cat*. Calli belonging to these two groups should be distinguishable from each other by any screen for the expression of *cat*, and the results of the screen should predict whether or not each individual callus will contain CAT activity when assayed. Therefore, to investigate the effectiveness of the proposed screen for resistance, each of 21 potential pLGV1103/pCAP212 cotransformants that had been maintained on MS plus kanamycin at 50 µg/ml were divided into five and one piece of each was transferred to plates containing MS plus chloramphenicol at 0, 10, 20, 30 or 40 µg/ml. As a negative control, 21 kanamycin resistant calli obtained from transformation with pLGV1103 alone were grown on the same series of media.

All calli began to grow within two weeks in the absence of chloramphenicol. At 10 µg/ml or above, the negative controls had not grown perceptibly after four weeks, but had lost their green colour and become pale or brown. Two weeks after transfer, the 21 potential cotransformants were separable into two groups. Eight (those designated a, e, g, i, j, o, r, and s) behaved like the negative controls, but 13 (those designated b, c, d, f, h, k, l, m, n, p, q, t, and u) produced new white or green callus at their surface on all chloramphenicol concentrations, and this distinction became more apparent in the third and fourth weeks. To check the reproducibility of the result, the portion of each of the 21 cotransformants and negative controls that had grown on MS medium without chloramphenicol was divided and replated on MS supplemented with 0, 40, 50 or 60 µg chloramphenicol /ml. The

same phenotype was shown by all calli during this second round of screening with the exception of one (callus b.) which failed to sustain growth on chloramphenicol. Cat assays were performed on 11 of these 21 calli to determine whether the resistant phenotype correlated with expression of the CAT gene. Tissue from callus grown in the absence of chloramphenicol was used to exclude the possibility of induction of endogenous activities in resistant lines during screening. CAT activities in all the resistant calli were higher than those in any of the sensitive calli which showed only backgroung levels of activity (Figure 6.8). Callus b. expressed no CAT activity in this assay, but when tissue that had been grown on chloramphenicol containing medium was tested, strong activity was detected. It is possible that in callus b, an induction effect was observed, but it is more likely that the tissue was originally genetically chimaeric, or that expression of *cat* was being modified in some parts of the callus (Czernilofsky *et al.* 1986b).

Several conclusions can be drawn from this experiment. Firstly, the screening procedure for chloramphenicol resistance is effective. In 10 of 11 cases it allowed successful prediction of whether or not calli would express *cat*. Secondly, it is highly discriminatory. Growth of calli that do not contain or do not express the CAT gene was inhibited by 10 μg/ml chloramphenicol whereas all chloramphenicol resistant calli grew unimpaired at chloramphenicol concentrations of up to 60 μg/ml. This can be compared to the poor discrimination obtained earlier when such calli are growing in agarose beads. Thirdly, callus k in table 6.2 showed low levels of CAT activity but its growth on all concentrations of chloramphenicol did not appear to be correspondingly reduced. This further supports the notion that this is a sensitive screen for resistance which will be effective in identifying true transformants if large numbers of marginally chloramphenicol resistant calli need to be screened in mitochondrial transformation experiments.

These initial conclusions have been substantiated in further experiments (for example Figure 6.10). All chloramphenicol resistant transformants obtained to date have grown on MS medium supplemented with chloramphenicol at 10μg/ml and have expressed CAT whenever assayed. Figure 6.9A shows that calli transformed with pCAP212 and selected with chloramphenicol were resistant to the antibiotic at up to 100 μg/ml in MS medium, although callus number 11 seemed to grow poorly above 10 μg/ml. Kanamycin resistant calli fell into two completely distinct groups that either showed CAT activity and were resistant or did not and were sensitive.

Figure 6.8

Chloramphenicol Acetyltransferase Activity in Kanamycin Resistant Calli Obtained from Cotransformation of pLGV1103 and pCAP212 and Screened for Resistance to Chloramphenicol.

As described in section 4.4.1, kanamycin resistant calli were recovered from transformation of *N. tabacum* cv. Petit Havana SR1 protoplasts with a mixture of pLGV1103 and pCAP212 using the PEG-calcium nitrate transformation procedure. Twenty one calli were screened for resistance to chloramphenicol at concentrations ranging from 10 to 60 µg/ml. The CAT activity demonstrated by eleven of these calli (a, b, c, d, e, f, g, h, i, k, and l) was determined and the results are compiled in this figure. Calli can be divided into two distinct groups that either contain elevated CAT activity and are chloramphenicol resistant, or show background levels of activity and are chloramphenicol sensitive. Calli a, b, c, d, e, f, g, and h are equivalent to calli designated CAP 6, 5, 4, 3, 7, 8, 9 and 10 respectively in Figure 4.3 A.

0, 1, and 3 indicate the position of chloramphenicol and its two monoacetylated derivatives.

Figure 6.8

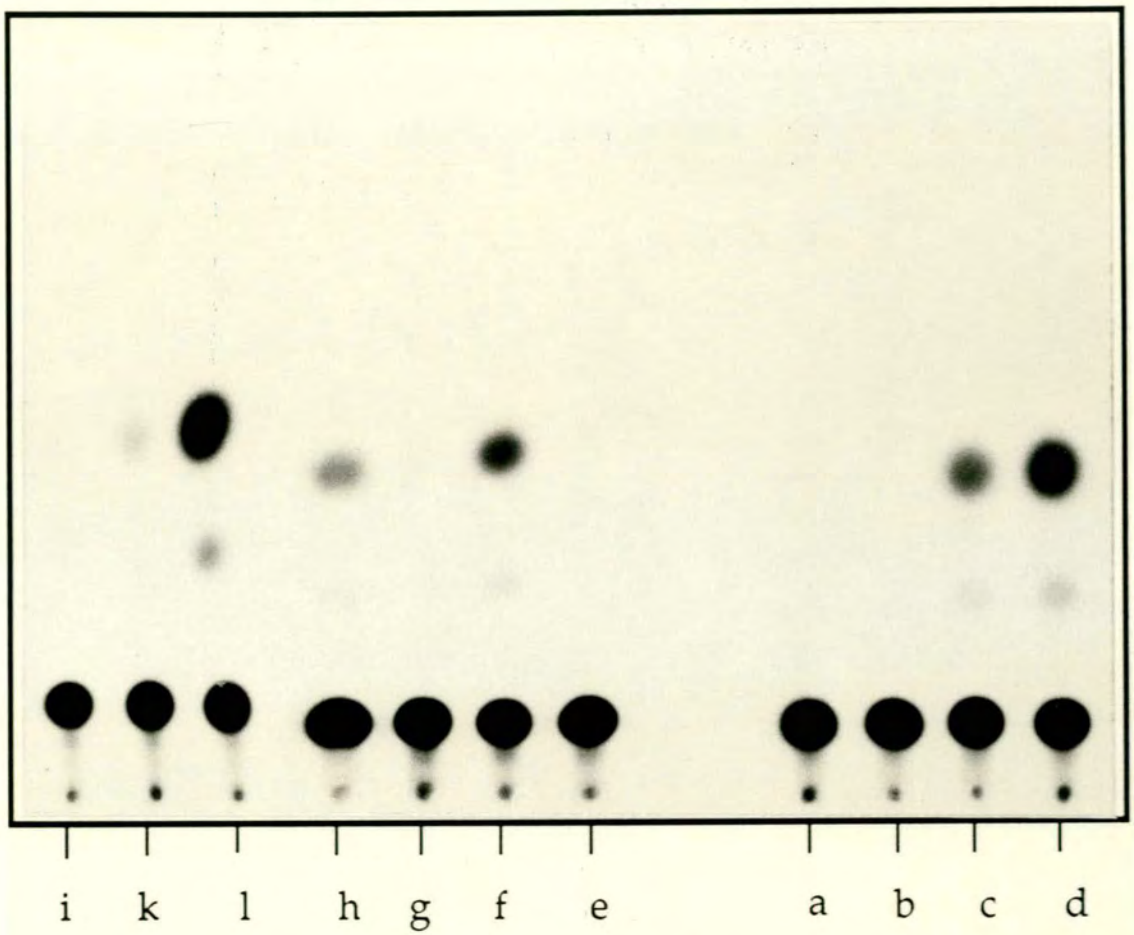


Figure 6.9 A

Screening Calli Selected for Resistance to Chloramphenicol or Kanamycin for Resistance to Each Antibiotic.

N. tabacum cv. Petit Havana SR1 mesophyll protoplasts were transformed with plasmids pCAP212, pLGV1103, or pRT-T1 using the PEG-calcium nitrate transformation procedure. Plasmids pLGV1103 and pRT-T1 allow recovery of kanamycin and chloramphenicol resistant calli respectively, and pCAP212 allows recovery of resistant calli using both antibiotics. Chloramphenicol and kanamycin resistant calli were selected from these protoplasts as appropriate using the standard selection regimes for each antibiotic.

Six kanamycin and thirteen chloramphenicol resistant calli recovered from the sample transformed with pCAP212 were transferred to Murashige and Skoog (MS) medium supplemented with agar and 50 µg kanamycin/ml or 10 µg chloramphenicol/ml as appropriate. Similarly, seven kanamycin resistant calli and four chloramphenicol resistant calli recovered from transformation with pLGV1103 and pRT-T1 respectively were transferred to similar medium.

Calli were grown for about three weeks and were then divided into 11 pieces and one piece of each callus was transferred to a series of petri dishes containing (MS) medium supplemented with chloramphenicol (Cm) at concentrations ranging from 0 to 200 µg/ml or with kanamycin (Km) at concentrations from 50 to 750 µg/ml. Calli were grown for three weeks on each antibiotic regime, then collected, arranged on larger petri dishes and photographed.

The plasmid used to generate each initial transformant, and the resistance phenotype that was initially selected (kanamycin, KmR, or chloramphenicol, CmR) are indicated at the top. Calli derived from each initial transformant and grown at different antibiotic concentrations are arranged in columns from top to bottom. The number of each transformant is shown along the bottom. The calli in each row were all grown at the same antibiotic concentration, and this is indicated at the right. The pRT-T1 and pLGV1103 derived transformants grown without antibiotic and the chloramphenicol resistant pCAP212 derived transformants grown on kanamycin at 100 µg/ml were lost due to fungal contamination.

Figure 6.9 A

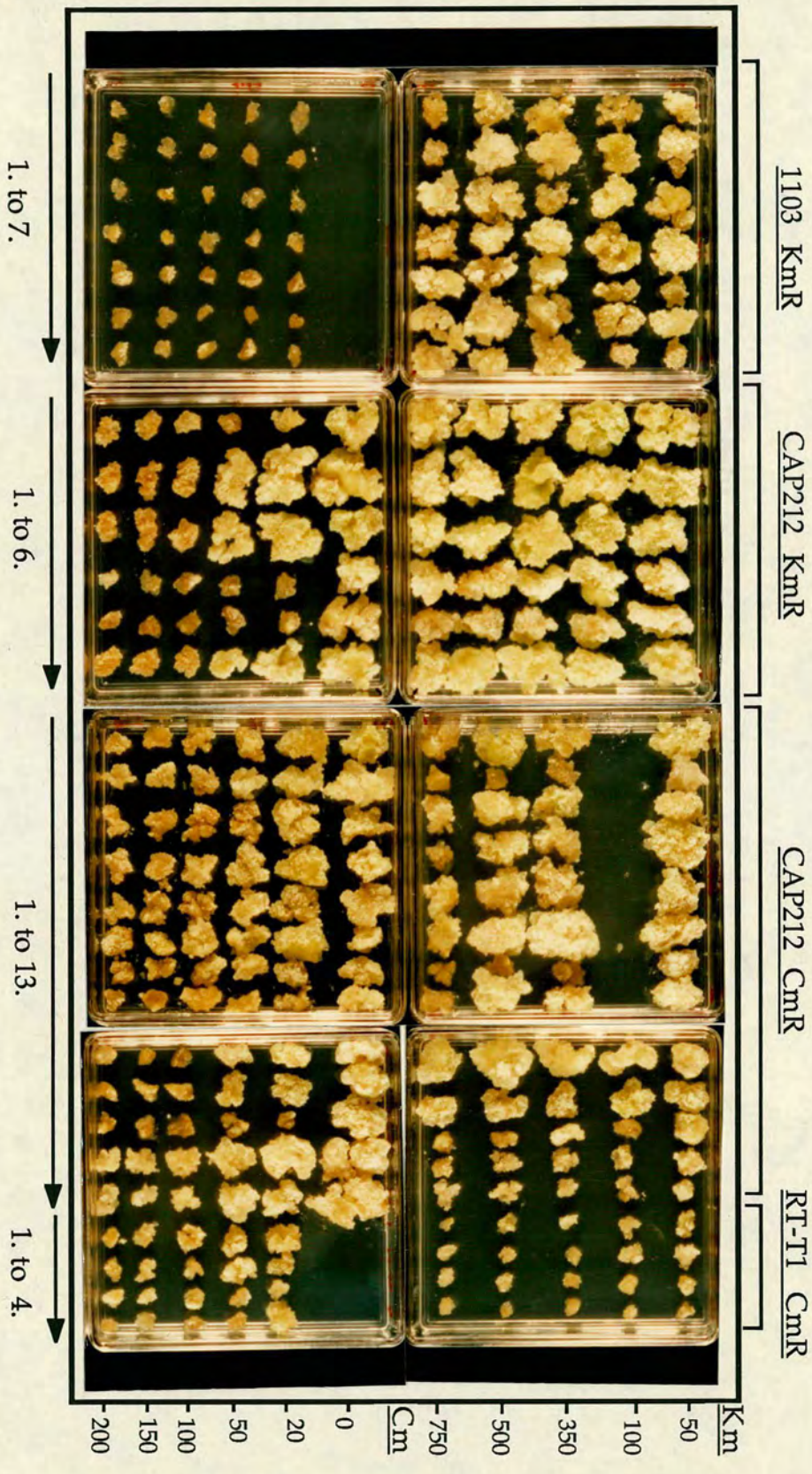

1103 KmR
CAP212 KmR
CAP212 CmR
RT-T1 CmR
Km
50
100
350
500
750
Cm
0
20
50
100
150
200
1. to 7.
1. to 6.
1. to 13.
1. to 4.

Figure 6.9 B

Chloramphenicol Acetlytransferase Activity in Chloramphenicol or Kanamycin Resistant Transformants Recovered Using pCAP212.

The pCAP212 derived transformants studied in the experiment described in Figure 6.9A were used for this assay.

The CAT activity in the chloramphenicol resistant (CmR) calli numbered 1 to 7 and 9 to 12 was assayed and is shown. Compared to untransformed tissue (SR1) all demonstrated high levels of activity. The CAT activity in chloramphenicol resistant calli numbered 8 and 13 was assayed separately; the result for callus 8 is shown in Figure 6.11B; that for number 13 is not shown, but was similar to the those of numbers 1 to 12.

All six kanamycin resistant (KmR) calli (numbers 1 to 6) were assayed in addition to five other kanamycin resistant calli (numbers 8 to 12) not included in the analysis in Figure 6.9A. Six transformants demonstrated CAT activity elevated above background (SR1), and five demonstrated activity similar to the background level. The expression of CAT activity correlated with the chloramphenicol resistant phenotype observed in Figure 6.9A.

0, 1 and 3 indicate the positions of chloramphenicol and its two monoacetylated forms.

Figure 6.9 B

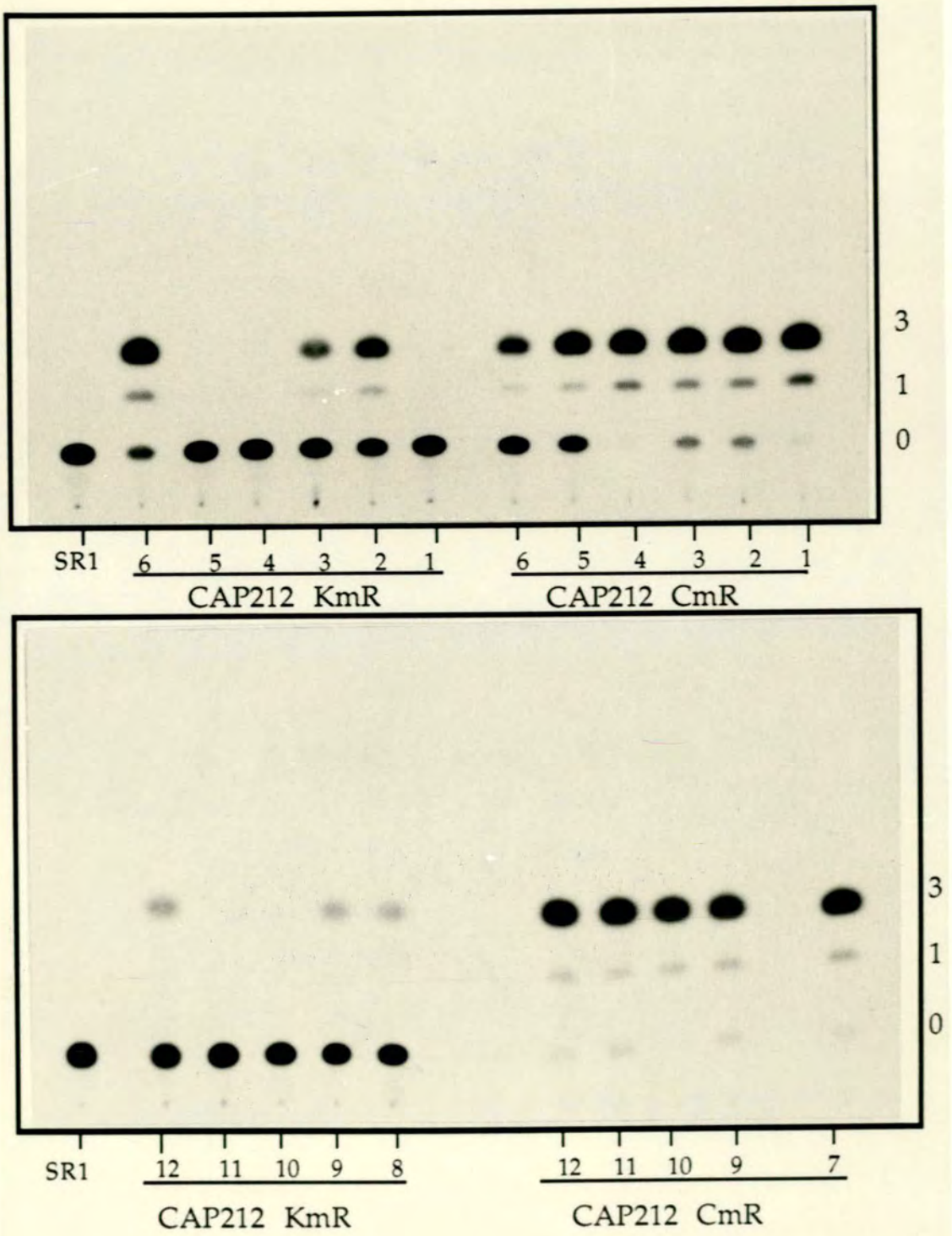

3
1
0
SR1
6 5 4 3 2 1
6 5 4 3 2 1
CAP212 KmR
CAP212 CmR
3
1
0
SR1
12 11 10 9 8
12 11 10 9 7
CAP212 KmR
CAP212 CmR

Figure 6.10

Screening Transformed Calli for Chloramphenicol and Kanamycin Resistance.

Some of the transformed calli studied in the experiment shown in Figure 6.9A were used in this experiment.

Approximately 50 mg of tissue from eight calli transformed with pCAP212 and originally selected for resistance to chloramphenicol (numbers 1 to 8) were transferred to a petri dish containing solidified Murashige and Skoog medium supplemented with chloramphenicol (Cm) at 10 µg/ml. A similar amount of tissue was transferred to a second petri dish containing the same medium supplemented with kanamycin (Km) at 50 µg/ml. Four chloramphenicol resistant calli transformed with pRT-T1 and seven kanamycin resistant calli transformed with pLGV1103 were similarly divided between the two plates. The arrangement of the transformants on each plate is illustrated by the diagram; the callus number, the plasmid with which it is transformed, and the selectable marker gene carried by the plasmid are indicated.

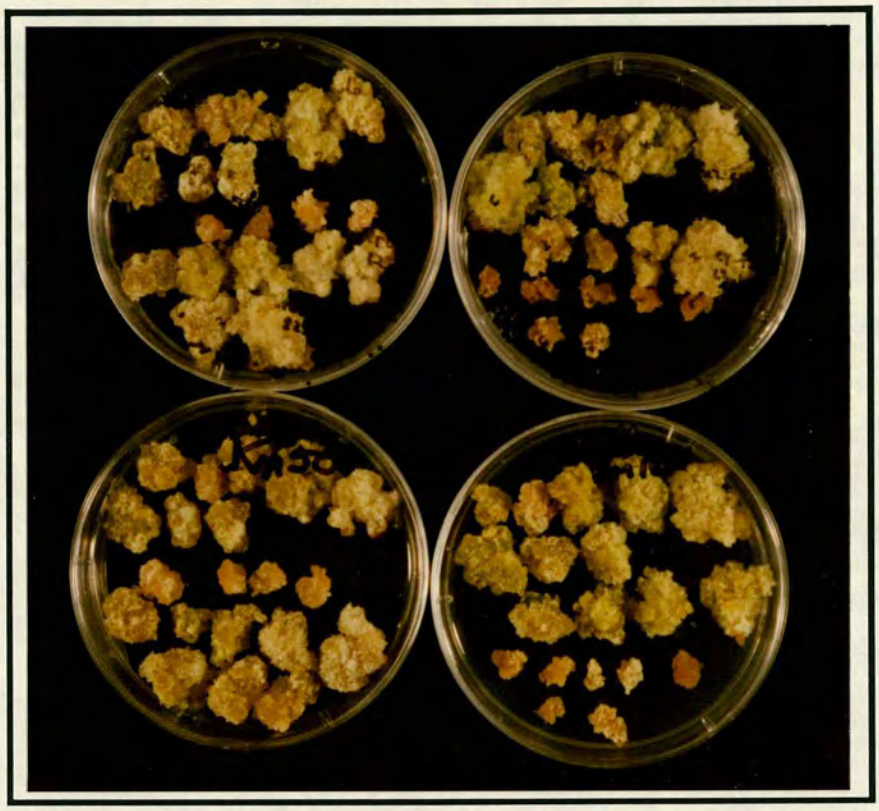
The calli were grown for approximately four weeks and photographed. Duplicates of each plate are shown to provide an indication of the variability in growth rates seen in tissue culture.

The calli transformed with pCAP212 grew on both chloramphenicol and kanamycin containing media; those transformed with pRT-T1 and pLGV1103 grew only on media containing chloramphenicol and kanamycin respectively.

Figure 6.10

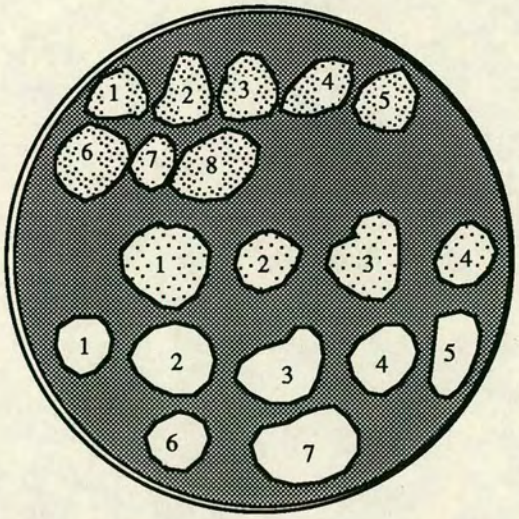
Km
50 µg/ml

Cm
10 µg/ml



Km
50 µg/ml

Cm
10 µg/ml



pCAP212 CmR CAT/NPTII

pRT-T1 CmR CAT

pLGV1103 KmR NPTII

The results reported in the preceding sections have shown that despite earlier suggestions (De Block *et al.* 1984, Pietrzak *et al.* 1986), *cat* in combination with chloramphenicol can be used reliably and easily to select transformed and regenerating protoplasts. This in effect provides an alternative selection system for genetic manipulation and analysis of plant cells. CAT is just as versatile as other markers, but easier, cheaper and more quantitative to assay than *nptII* which currently is most frequently used. After selection, the transformed phenotype can be confirmed by a sensitive screen for resistance on solidified MS medium. Secondly, all the experimental requirements of the proposed mitochondrial transformation strategy that are open to investigation have now been evaluated. As reported in previous chapters, evidence has been presented that *cat* in the mitochondrial transformation vectors is not expressed in plant cell nuclei and that it would probably be expressed poorly in their chloroplasts. It has now been shown that a derivative of CAT that is targeted to the mitochondria of transformed plant cells can confer a chloramphenicol resistant phenotype and that such chloramphenicol resistant nuclear transformants can be selected. However doubts remain that in practice this selection step may not be efficient enough to recover mitochondrial transformants, and that such cells may not express sufficient CAT activity to survive the selection procedure. These doubts and others concerning the design of the transformation vectors could be assessed further only by carrying out transformation experiments, and these initial experiments are described in the next section.

6.6 Preliminary Attempts to Obtain Mitochondrial Transformants.

At the end of section 6.5, it was concluded that the transformation strategy had been experimentally evaluated as far as was reasonable. Although doubts remain about some features of the plasmid design and about the efficiency of the selection procedure, these could not easily be evaluated further or be circumvented. Transformation experiments were initiated using the mitochondrial transformation vectors without further modification to the system. The main transformation effort is still to come and will be necessary for a proper evaluation of the system; however the results of the first experiments are briefly reported below.

The PEG-calcium nitrate procedure has been used to transform *N. tabacum*

cv. Petit Havana SR1 protoplasts with all the mitochondrial transformation vectors described in previous chapters. In a series of experiments, 40 μg of plasmid DNA was digested with a restriction endonuclease to generate linear molecules, mixed with 40 μg of supercoiled DNA of the same plasmid and the mixture used to transform $1x10^6$ protoplasts as described in Materials and Methods. As a positive control, every experiment included one sample transformed with 40 μg of pCAP212 (a nuclear transformation vector providing kanamycin and chloramphenicol resistance) which had been linearised at its *Hin*dIII site. This sample was then divided into two, and one portion selected with chloramphenicol, and the other with kanamycin. Plasmid pUPS92E and its various derivatives were used as negative controls in each experiment.

In most experiments, no chloramphenicol resistant calli were obtained, but as the transformation efficiency with the positive control in these cases was poor little could be drawn from these observations. In one experiment involving the plasmids p92J-T13, p92E-T13 and p92O-T13 potential mitochondrial transformants were observed. The positive control, pCAP212 yielded 25 kanamycin resistant calli (50 per 10^6 protoplasts), but no chloramphenicol resistant ones, and similarly, the sample transformed with p92J-T13 yielded no chloramphenicol resistant calli. In contrast, in samples transformed with p92O-T13 though no clearly resistant calli emerged several appeared to be growing slightly faster than the rest, and in the sample transformed with p92E-T13 three calli were distinctly greener than the others.

To continue the selection under less stringent conditions the three potential p92E-T13 transformants and 114 potential p92O-T13 transformants were transferred to MS medium supplemented with 10 μg chloramphenicol /ml. Within three weeks all three calli from the p92E-T13 transformation had grown vigorously. Even after four weeks, none of the other potential transformants had grown similarly to these three nor had they grown as well as the nuclear transformants recovered previously.

A CAT assay was performed on the three apparently resistant p92E-T13 derived calli and on the seven least inhibited p92O-T13 derived calli. As shown in Figure 6.11B, all these calli expressed activities similar to or less than the negative control, the pLGV1103 transformant, and far less than the positive control, the pRTpreβcat transformant. It is of interest that the activity in all potential transformants obtained with p92O-T13 was higher than in all those obtained with p92E-T13, as would be expected on the basis of expression from the *cox1* promoter. The background CAT activity observed with the pLGV1103 transformant and uncertainty about the level of activity to be expected in genuine mitochondrial transformants make these results difficult to interpret. This, coupled with the recovery of resistant calli using p92E-T13 provided no evidence for transformation. All calli originally transferred to MS medium were replated

on chloramphenicol to determine the reproducibility of the resistant or sensitive phenotype, and to generate material for molecular analysis if necessary. The resistant phenotype was found not to be consistently sustained by any of the calli during successive subcultures (Figure 6.11A).

In other experiments three marginally resistant calli were obtained with pUPS92J (Figure 6.11B), two with p92E-T13, three with pAP-S13 and three with pAP-S13.9. None of these have been found to express CAT activities above background (not shown), and continued growth was observed with only one p92E-T13 derived and one pAP-S13 derived callus. Again the resistance to chloramphenicol was marginal with no evidence for transformation being obtained.

The most promising potential transformant was obtained using p92O-S13. The positive control in this experiment, pCAP212, yielded 16 chloramphenicol resistant calli per million protoplasts. No potential transformants were recovered when p92J-S13 or p92E-S13 were used. In the sample transformed with p92O-S13 two calli emerged which were distinctly greener and larger than the rest, and one of these showed sustained growth on MS plus chloramphenicol at 10 μg/ml, though was slow to grow on 20 μg/ml. This line has shown continued resistance over several subcultures, but significantly elevated CAT activity could not be detected (Figures 6.12 and 6.11B). Plants are being regenerated and Southern blot hybridisation analysis performed to try and establish whether transformation has occurred.

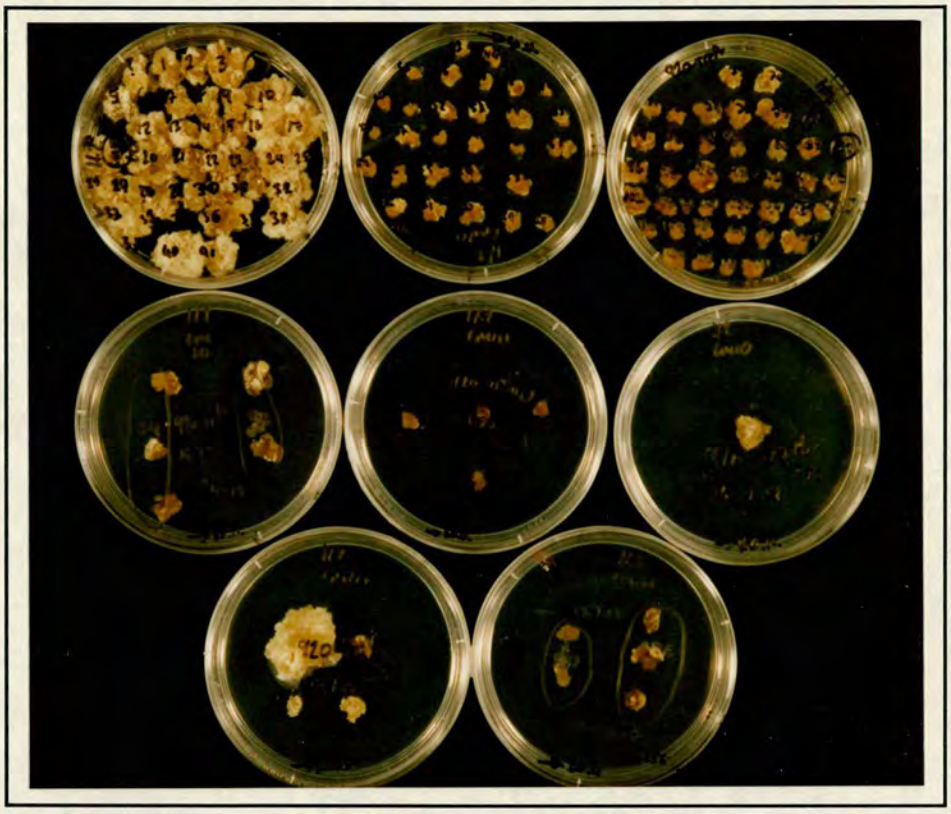
Figure 6.11A

Screening Potential Mitochondrial Transformants for Chloramphenicol Resistance.

Potential mitochondrial transformants were recovered by transformation of *N. tabacum* cv. Petit Havana SR1 protoplasts with p92O-T13 using the PEG-calcium nitrate transformation procedure as described in the text. 114 such calli were transferred to petri dishes containing solidified Murashige and Skoog medium supplemented with chloramphenicol at a concentration of 10 μg/ml. After approximately six weeks, the calli were transferred to a second set of petri dishes containing the same medium. Those calli that had grown sufficiently were divided into several pieces upon transfer; those that had not grown sufficiently to be divided were transferred in one piece. After a second period of culture, the calli were transferred to a third set of plates as before, and photographed after about five weeks. Each callus was given a number, and the arrangement of the calli on the petri dishes at this stage is illustrated by the diagram below the photograph.

Initially calli number 4, 19, 62, 73, 76, 82, and 86 appeared to be growing significantly faster than the remainder. At the first transfer, number 62 was divided into four as it had grown most rapidly, however these pieces showed no further growth during the two subsequent culture periods. During the second culture period, calli number 4, 19, 82, 86 and particularly 76 showed most rapid growth, and were divided at the second transfer. However of these only one piece of callus (derived from number 76) sustained growth as shown, and by this stage calli number 1 to 41 had grown similarly. None of the calli showed sustained chloramphenicol resistance.

Figure 6.11 A



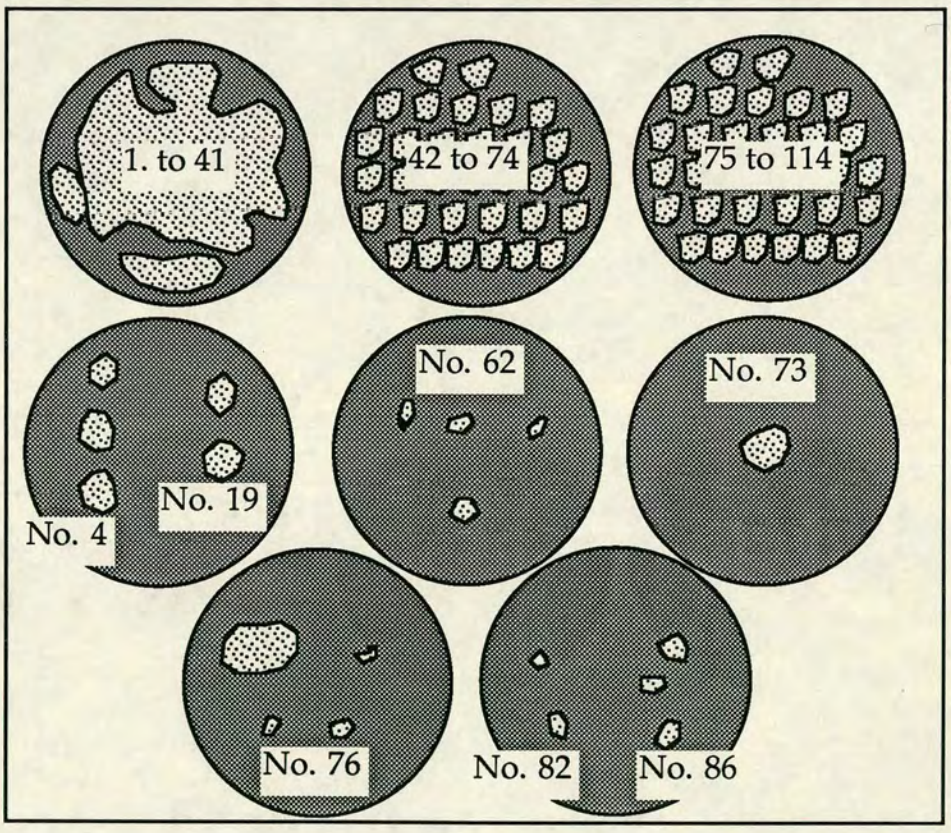


Figure 6.11 B

Chloramphenicol Acetyltransferase Activity in Potential Mitochondrial Transformants.

i. The CAT activity demonstrated by the seven potential transformants obtained with p92O-T13 (numbered 4, 19, 73, 62, 76, 82 and 86) that were described in the text and in Figure 6.11A is shown. The three chloramphenicol resistant calli that arose in the same experiment from transformation with p92E-T13 (1, 2 and 3) were also assayed. The CAT activity demonstrated by callus transformed with pLGV1103 (1103) and pRTpreβcat (β) was assayed to provide controls. CAP shows a sample of the radioactive chloramphenicol added to the assay.

ii, iii, and **iv.** CAT activity displayed by each of three potential mitochondrial transformants obtained using pUPS92J (f, g, and h) and two obtained using p92O-S13 (1 and 2) is shown. Clone number 1 of the latter two has been assayed on a number of occasions, and its growth on chloramphenicol is shown in Figure 6.12. Positive controls were leaf tissue from a tobacco plant (JV) transformed with pCAP212 which was known to express weak CAT activity (see Figure 4.3), and callus tissue also transformed with pCAP212 (number 8) which displays greater CAT activity. Negative controls were untransformed tobacco leaf tissue (SR1), or callus tissue transformed with plasmid pLGV1103 (1103) that does not contain *cat* . CAP shows a sample of the radioactive chloramphenicol added to the assay.

The only potential transformant to display greater CAT activity than the negative controls was p92O-S13 number 1 (**ii.**). However, the activity was weak and not displayed in the other assays of this clone, so its significance is unclear.

Figure 6.11 B

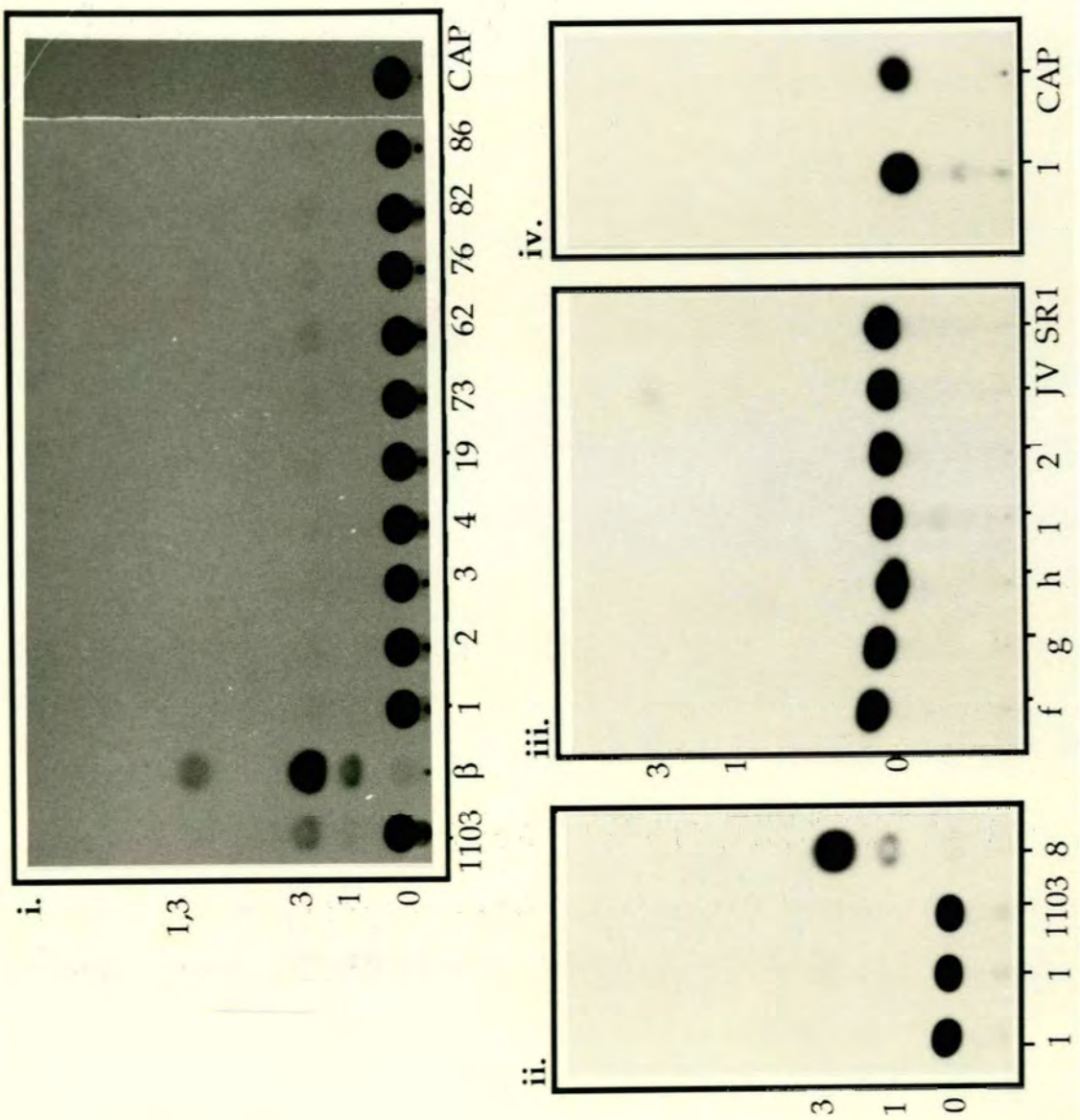

i.
1,3
3
1
0
1103 β 1 2 3 4 19 73 62 76 82 86 CAP
ii.
3
1
0
1 1 1103 8
iii.
3
1
0
f g h 1 2' JV SR1
iv.
1 CAP

Figure 6.12

Chloramphenicol Resistance of a Potential Transformant Recovered Using p920-S13.

A potential mitochondrial transformant, 92O-S13#1, was obtained by transforming mesophyll protoplasts of *N. tabacum* cv. Petit Havana SR1 with a mixture of linearised and supercoiled p92O-S13 using the PEG-calcium nitrate procedure (Materials and Methods). This callus has shown sustained slow growth on chloramphenicol but no significant CAT activity (Figure 6.11B).

Three pieces of approximately 50 mg of 92O-S13#1 (92O) were transferred to a petri dish containing solidified Murashige and Skoog medium supplemented with chloramphenicol at 10 µg/ml. Three pieces of a chloramphenicol resistant callus transformed with pRT-T1 (RT-T1) and a chloramphenicol sensitive callus transformed with pLGV1103 (LGV1103) were also transferred. The calli were photographed after approximately four weeks. The growth shown by all three pieces of 92O-S13#1 callus was intermediate between the growth of callus of the other two clones.

Figure 6.12

These initial experiments were inconclusive, but suggested at least that, when using this DNA delivery system and selection strategy, mitochondrial transformants are recovered less frequently than nuclear transformants. Similar transformation experiments are being repeated, and should eventually provide a realistic comparison of the relative ease of mitochondrial and nuclear transformation using the present strategy, though such a comparison based on the present limited results would be premature.

My inability to detect elevated CAT activity in the potential mitochondrial transformants does not support the proposal that they arose as a result of *cat* acquiring a promoter following its integration into the nuclear genome, or through induction of an endogenous CAT activity. It also increases confidence in the CAT assay as a reliable indicator of at least nuclear transformation. All the potential mitochondrial transformants selected above showed such marginal resistance that they would not have been considered resistant in nuclear transformation experiments. Thus their recovery does not contradict earlier claims for the usefulness of *cat* as a nuclear resistance marker.

Most of the calli analysed in these experiments were probably false positives because they failed to sustain growth on chloramphenicol, though the origin of resistance in those that continued to grow is unclear. Plants are being regenerated from these calli to further investigate the source of their resistance. In the mitochondrial transformation experiments they were analysed only because genuine mitochondrial transformants may display similarly poor chloramphenicol resistance so such calli could not be ignored. The disadvantage in not having a positive control for mitochondrial transformation is demonstrated in these experiments.

The experiments reported here represent only the begining of a continuing attempt to use the plasmids that I have constructed in mitochondrial transformation experiments. Increasingly, attention must be paid to the types of DNA delivery system to be used, to the physiological state of the target tissue, and perhaps still to the culture medium used during selection. Various modifications to the strategy that have been initiated are described in Chapter 7, and potential developments for the future are discussed in Chapter 8.

CHAPTER 7

ALTERNATIVE SYSTEMS FOR DELIVERING DNA TO THE MITOCHONDRIA AND FOR RECOVERING TRANSFORMANTS.

7.1 Introduction

Initial attempts to obtain mitochondrial transformants involved direct DNA uptake by isolated protoplsts. The only successful mitochondrial transformation system reported to date involved the use of high velocity, DNA coated, tungsten microprojectiles which were used to transform yeast cells (Johnston *et al.* 1988). It is now intended to use this delivery system in attempts to obtain plant mitochondrial transformants in the near future (Chapter 8), however at the time these experiments were being performed, the equipment was not available. Mitochondria of yeast were transformed 1000 fold less frequently than the nuclei when using high velocity microprojectiles, the most effective DNA delivery system yet applied to mitochondrial transformation. The possibility that a similar differential may exist in transformation of plant mitochondria, coupled with the relatively low efficiency of recovery of nuclear transformants with chloramphenicol, suggests that large numbers of plant protoplasts and potential transformation events may be required to recover a mitochondrial transformant. Cocultivation of protoplasts with *A. tumefaciens* has previously been reported to give the most consistently high nuclear transformation efficiencies; between 1 and 25% of treated protoplasts (for example Depicker *et al.* 1985, Velten and Schell 1985) compared to 0.01% for most other techniques (Fromm *et al.* 1986, Hain *et al.* 1985, Czernilofsky *et al.* 1986a, Wirtz *et al.* 1987). It is not clear which of the available transformation techniques is most suited to transfer DNA into mitochondria (but this is discussed in section 8.5), however the ease with which DNA can be transferred into large numbers of plant cells by the cocultivation technique gives it an advantage over the rest. For this reason, and in collaboration with Dr. C. Bachem, Ti plasmid derivatives carrying the mitochondrial selectable markers have been constructed, and transformation experiments initiated.

7.2 Transformation using *Agrobacterium tumefaciens*

7.2.1 Construction of Positive Control Plasmids.

Initially, I chose the binary vector pPCV706 (Koncz and Schell 1986, and C. Koncz personal communication) to construct Ti plasmid derivatives that would express *cat* from the nucleus and thus act as positive controls in transformation experiments (Figure 7.1). This binary vector contains an origin of replication of the ColE1 type and origins of vegetative and conjugal replication from the RK2 plasmid to facilitate its replication in both *E. coli* and *A. tumefaciens* and its transfer between these two bacterial species. It expresses a β-lactamase gene providing resistance to both ampicillin and carbenicillin thus allowing its selection and maintenance in bacteria. It also contains the 25 bp border repeats derived from the Ti plasmid that are required in *A. tumefaciens* to transfer the T-DNA to plant cells. The small size of the vector (less than 10 kb) allows easy manipulation using the usual *in vitro* recombination techniques. Thus a sequence of interest can be ligated into this plasmid between the T-DNA borders and the desired recombinants identified following transformation into *E. coli.* Strain S17-1 is used because it possesses all the genetic functions necessary for conjugal transfer of pPCV706 into the *A. tumefaciens* strain GV3101-MP90RK. This *A. tumefaciens* strain, unlike *E. coli* strain S17-1, is resistant to rifampicin and kanamycin, so after conjugation between these two strains, GV3101 MP90RK clones carrying the pPCV706 derivative can be selected for by growth on carbenicillin and rifampicin. The chromosome and Ti plasmid of this strain possess the necessary *chr* and *vir* functions to facilitate transfer of T-DNA to plant cells, however the wild type T-DNA has been deleted from the resident Ti plasmid and the transfer functions act solely on the 25 bp border repeats of pPCV706 and the sequences of interest between them.

The promoter, CAT gene and polyadenylation signal of plasmids pRT-T1 and pRTpreβcat were inserted at the unique *Eco*RI and *Hin*dIII sites between the T-DNA borders of pPCV706 to produce the plasmids pPCRT-T1 and pPCRTβcat. The subcloning strategy designed for this purpose is shown in Figure 7.1, and plasmid construction was performed by Dr. C.W.B. Bachem. Initially these sequences were isolated as *Pst*I fragments from pRT-T1 and pRTpreβcat, and inserted into the polylinker of pK18 to introduce an *Eco*R1 site 3' of the polyadenylation signal. Plasmids pPCRT-T1 and pPCRTβcat were constructed by sequential transfer of *Hin*dIII and *Eco*RI fragments from each of the pK18 clones into the equivalent sites of pPCV706.

Figure 7.1

Plasmid pPCV706 and Construction of pPCRT-T1 and pPCRTβcat.

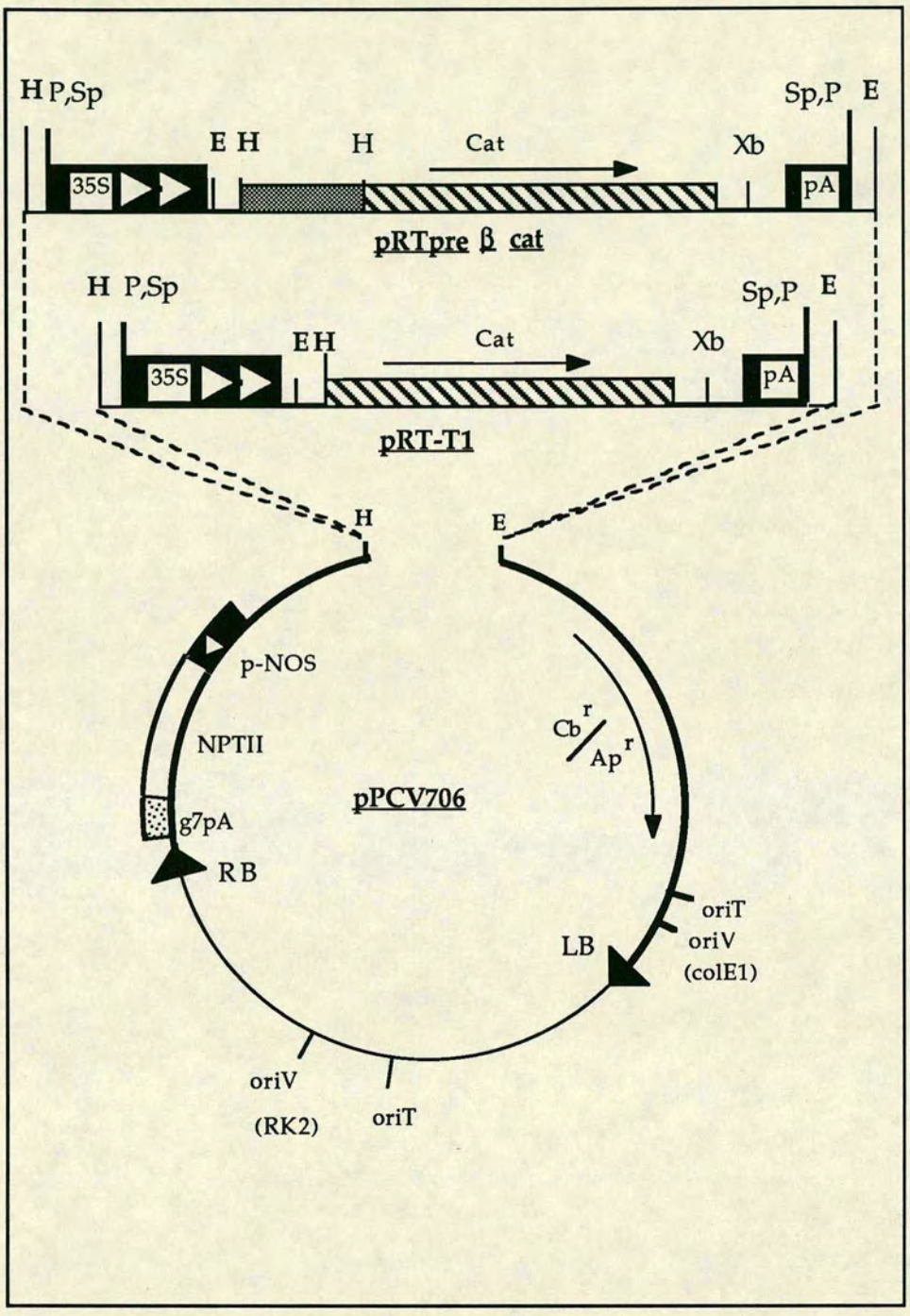
Plasmid pPCV706.

Plasmid pPCV706 is shown schematically at the bottom of the figure. The sequence between its*Hin*dIII (H) and *Eco*RI (E) recognition sites which were used in subsequent cloning steps has been omitted for simplicity. The plasmid is based on the RK2 wide host range replicon that allows maintenance in *A. tumefaciens* and *E. coli* (oriV) and conjugal transfer between these species (oriT). Plasmid pPCV706 carries only some of the RK2 genetic functions necessary for its replication and transfer, the remainder being provided by the *E. coli* and *A. tumefaciens* host strains S17-1 and GV3101 MP90RK that are used with this plasmid. It also has an origin of replication of the ColE1 type that can replicate to high copy number in *E. coli*, but is inactive in *A. tumefaciens*. The β-lactamase gene provides resistance to ampicillin (Ap^r) in *E. coli* and to carbenicillin (Cb^r) in *A. tumefaciens*. The left and right T-DNA borders are shown as black triangles labelled LB and RB, and the T-DNA sequence between them is indicated by the heavy line. As a selectable marker for plant transformation, the T-DNA carries a neomycin phosphotransferase gene (NPTII) expressed from the nopaline synthase gene promoter (p-NOS) and the T-DNA gene 7 polyadenylation signal (g7pA); this chimaeric marker confers kanamycin resistance.

Construction of pPCRT-T1 and pPCRTβcat.

The promoter, CAT gene and polyadenylation signal were isolated from each of pRT-T1 and pRTpreβcat as *Pst*I fragments (see Figure 6.6A), and inserted into the *Pst*I site (P) in the polylinker of pK18 (identical to the polylinker of pUC19, Pridmore 1987). These derivatives are shown at the top of the figure; each one contains a *Hin*dIII site adjacent to the 35S promoter, and an *Eco*RI site downstream of the polyadenylation signal. Initially the *Hin*dIII fragments from these plasmids were introduced into the unique *Hin*dIII site of pPCV706; the *Eco*RI fragments of the same plasmids were then inserted between the *Eco*RI sites situated in the transferred *Hin*dIII fragment and in the pPCV706 sequence. The net result was that the *Hin*dIII to *Eco*RI fragment from each pK18 derivative was inserted between the *Hin*dIII and *Eco*RI sites of pPCV706. Sp, *Sph*I; Xb, *Xba*I.

Figure 7.1


H P,Sp
Sp,P E
E H
H
Cat
Xb
35S
pA
pRTpre β cat
H P,Sp
Sp,P E
EH
Cat
Xb
35S
pA
pRT-T1
H
E
p-NOS
NPTII
g7pA
RB
pPCV706
Cb r / Ap r
oriT
oriV
(colE1)
LB
oriV
(RK2)
oriT

7.2.2 Construction of Ti Plasmid Derived Mitochondrial Transformation Vectors.

Plasmid pBIN19 (Bevan 1984), a binary vector similar to pPCV706 was used for construction of Ti plasmid derived mitochondrial vectors. This plasmid differs from pPCV706 in that it has replication and transfer functions from only RK2, and that it confers kanamycin resistance, but not ampicillin or carbenicillin resistance, to the bacterial host. The polylinker and α-complementation region of M13mp18 is carried between its T-DNA borders therefore insertion of additional DNA sequences at the polylinker inactivates β-galactosidase, allowing the insertion to be screened for with X-gal. The 2.4 kb *Pvu*II to *Hin*dIII fragment was isolated from plasmids p92J-T13, p92E-T13 and p92O-T13. This fragment extends from the *Pvu*II site within the vector sequence upstream of the COXI promoter, through the entire chimaeric CAT gene to the *Hin*dIII site downstream of the T13 sequence (Figure 5.4B). Each fragment was ligated into the *Sma*I and *Hin*dIII sites of pBIN19 to generate plasmids pBIN92J, pBIN92E and pBIN92O respectively (Figure 7.2). A similar plasmid is being constructed with the *Petunia atp9*-1 promoter in place of the maize *coxI* promoter.

The T-DNA of all these plasmids contains an NPTII gene under control of the nopaline synthase promoter and the octopine synthase polyadenylation signal. This chimaeric gene provides selectable kanamycin resistance to plant cells and can be used as an internal control in transformation experiments.

7.2.3 Transformation of Plant Cells with Ti Plasmid Derived Transformation Vectors.

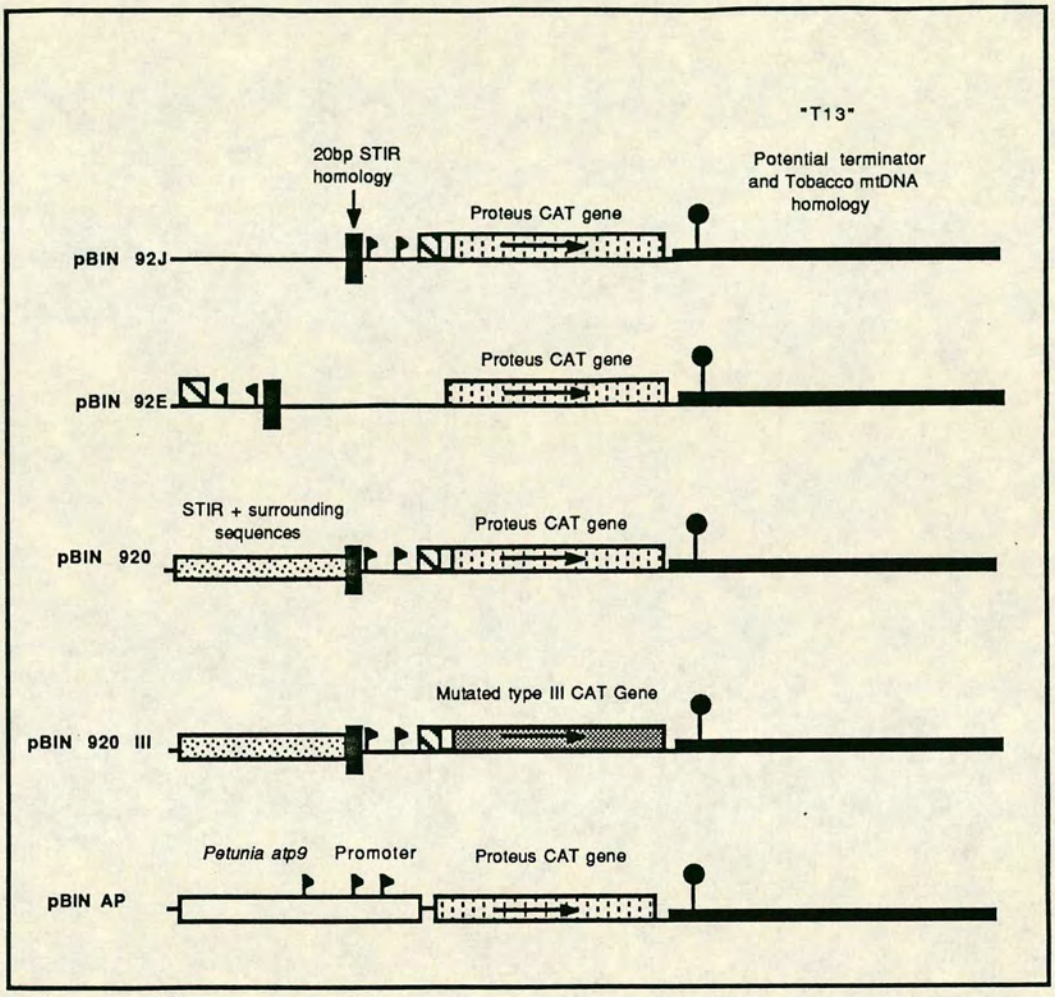
I used the positive controls pPCRT-T1 and pPCRTβcat in experiments to establish procedures for transformation of *N. tabacum* cells with *Agrobacterium* . Tobacco leaf disc transformation was used to evaluate the efficacy of the two nuclear selectable markers pPCRT-T1 and pPCRTβcat, and then a procedure for cocultivation was established.

7.2.3.1 Leaf Disc Transformation.

This technique was first reported by Horsch *et al.* (1985), and involves the induction of transformed shoots on excised leaf pieces infected with *Agrobacterium*. Rooted transgenic plants can be obtained in about 6 weeks making it the simplest and most rapid transformation procedure available to date. Successful use of *cat* as a selectable marker in this procedure would enhance its utility as a general marker for transformation. In leaf disc transformation

Figure 7.2

Binary Ti-Plasmid Vectors for Mitochondrial Transformation.



This figure shows schemetic diagrams of the chimaeric gene constructs introduced into the polylinker of pBIN19 to produce binary vectors for mitochondrial transformation. The origins of the promoter and CAT gene in each construct are indicated. All plasmids have been drawn according to the schemes used in previous diagrams. All *in vitro* manipulations involved in the construction of these plasmids were performed by Dr. C.W.B. Bachem.

protocols, selection is performed on solidified antibiotic supplemented MS medium. This medium was found to be particularly effective for identification of transformed chloramphenicol resistant calli (section 6.5). In initial experiments (not shown) *N. tabacum* leaf pieces failed to initiate shoots after one week on more than 5 µg chloramphenicol/ml, but pieces from leaves of a plant transformed by pCAP212 continued to shoot on media containing chloramphenicol at 20 µg/ml, suggesting that selection on this medium may be similarly effective for leaf disc transformation

Several protocols for transformation have now been published, but that of Draper *et al.* (1988) was used in these experiments. Leaves were cut in turn from 6 sterile *N. tabacum* cv. Petit Havana SR1 plants. Following removal of the midrib leaves were cut into pieces about $1cm^2$ and distributed between four petri dishes containing MS medium supplemented with 1 µg/ml BAP and 0.1 µg/ml NAA. On the same day single colonies of GV3101-MP90RK harbouring pPCRT-T1 or pPCRTβcat were used to inoculate 5 ml of Luria Broth containing rifampicin and carbenicillin at 100 µg/ml. Similar cultures were initiated with a colony of GV3101-MP90RK selected with 100 µg/ml of rifampicin, and one of *A. tumefaciens* strain LBA4404 harbouring the vector pBIN19 and selected with rifampicin and kanamycin at 100 µg/ml, and streptomycin at 300 µg/ml. This later strain was included to compare the transformation efficiency of the pBIN19 and pPCV706 vector and host systems. After two days shaking at 28 °C stationary phase cultures were obtained, diluted 20 fold in the same medium and grown for 12 to 14 hours to late log phase. Each of these was diluted 50 fold in MS medium and into this the leaf pieces from one petri dish (about 10 pieces) were dipped, blotted on Whatman 3MM paper and returned to their original position on the dish. After three days at 25 ^{0}C, bacteria had grown around the leaf pieces which were transferred to new media as follows: each sample was divided between plates containing the above medium additionally supplemented with 500 µg/ml Claforan to prevent further bacterial growth, and either chloramphenicol at 20 µg/ml or kanamycin at 50 µg/ml to select the transformants.

As a control, a few leaf pieces were cultured in the absence of either of the two antibiotics used for selection, and within two weeks a proliferation of shoots and callus from their cut edges was observed as expected. All the leaf discs were then transferred to fresh medium containing the appropriate antibiotics, and four weeks after infection the number of transformed shoots was estimated using a binocular microscope. Several morphologies of more or less teratomatous outgrowth result at the wound sites, but only those bearing two or more distinct leaves were scored (Table 7.1).

The emergence of shoots during selection with each antibiotic from discs inoculated with the negative control GV3101-MP90RK that lacks T-DNA suggests that the antibiotic concentrations in the medium were too low. However far more

shoots resulted when an appropriate selectable marker was used for transformation, implying that transformation had been successful. Several authors (for example DeBlock *et al.* 1984, Horsch *et al.* 1985) have reported that formation of roots under antibiotic selection is a reliable indicator of the antibiotic resistance of the shoot. This observation was confirmed in this work using wild type and pCAP212 transformed tobacco (not shown). Shoots derived from Experiment 1, Table 7.1, were transferred to MS medium supplemented with 500 µg Claforan /ml and either 10 µg chloramphenicol /ml (DeBlock *et al.* 1984) or

Table 7.1
Recovery of Antibiotic resistant Shoots Emerging from Transformed Leaf Discs.

Experiment	Antibiotic	RT-T1	βcat	BIN19	GV3101
1	Km	55	29	38	16
	Cm.	23	18	5	8
2	Km	27	23	-	8
	Cm.	23	10	-	9
3	Km	31	17	19	4
	Cm.	4	1	2	3
Mean	Km	38	23	29	9
±0.5	Cm.	17	10	4	6

The number of resistant shoots recovered from three separate leaf disc infections is shown. Leaf discs were infected with either *Agrobacterium* alone (GV3101), this strain harbouring either pPCRT-T1 or pPCRTβcat (RT-T1 and βcat, respectively), or strain LBA4404 harbouring pBIN19 which contains a nuclear kanamycin resistance marker, but no chloramphenicol resistance marker. Selection was performed on MS medium supplemented with either chloramphenicol (Cm) at 20 µg/ml, or kanamycin (Km) at 50 µg/ml.

100 µg kanamycin/ml. After about 10 days roots developed on about 40% of shoots derived from transformation with an appropriate selectable marker but not on those derived from the negative controls (Table 7.2). This confirmed that the shoots that had emerged from the negative controls were non-resistant escapes. This probably accounts in part for the low frequency of rooting amongst the potentially transformed shoots from the other samples. CAT assays on several

of the chloramphenicol resistant, rooted transformants showed high levels of activity in each as expected (Figure 6.7).

The relative efficiency with which transformants were recovered using the cytosolic and mitochondrially targeted CAT is very similar in this experiment to that observed previously with the PEG-calcium nitrate transformation technique (section 6.4). The results obtained with pPCRTβcat and pPCRT-T1 are thus consistent with those obtained with pRTpreβcat and pRT-T1. In these leaf disc transformation experiments, when pPCRTβcat was used, fewer kanamycin resistant shoots were recovered than when pPCRT-T1 was used. As discussed in section 6.4, such an observation could potentially indicate that the different intracellular location of CAT was not entirely responsible for the difference observed in the recovery of chloramphenicol resistant shoots with these plasmids. However, in the data in Table 7.1 the difference in the mean frequency of recovery of apparently kanamycin resistant shoots using each plasmid is significant at less than the 90% level.

There appear to be more nonrooting escapes following transformation with a suitable plasmid bearing *A. tumefaciens* strain than there are in the negative controls. Perhaps this is due to cross protection from transformed cells or from bacteria expressing the antibiotic resistance genes. However, genuine transformants do appear to have been selected.

Subsequently, culture of leaf discs on chloramphenicol at 30 µg/ml and kanamycin at 100 µg/ml has been found to eliminate the emergence of shoots in the negative controls (Figure 7.3). Under these conditions, *cat* can be effectively used as a selectable marker for transformation. This system has now been used in initial transformation experiments with plasmids pBIN92J, pBIN92E and pBIN92O, and roots have initiated on the first selected shoots.

Despite the rapidity of the leaf disc transformation procedure, it generates relatively low numbers of transformants (10 to 100 per experiment). If the results of the initial leaf disc transformations indicate that mitochondrial transformation by *Agrobacterium* is not frequent, then cocultivation will be used as a more prolific source of transformants. Having shown that plants transformed with pPCRTβcat could be be selected in the leaf disc transformation system, this plasmid was used to establish a suitable procedure for transformation of protoplasts by cocultivation with *A. tumefaciens* .

7.2.3.2 Cocultivation of Protoplasts with *A. tumefaciens* Containing pPCRTβcat.

The CAT gene contained in pPCRTβcat allows recovery of chloramphenicol

resistant calli or plantlets following direct DNA transfer into protoplasts and also after leaf disc transformation. Although it is the least effective of the nuclear chloramphenicol resistance markers that have been used to date, it is perhaps the most significant for selection of mitochondrial transformants. The number of transformants recovered following selection with chloramphenicol and kanamycin can be directly compared as the plasmid also encodes a selectable NPTII gene conferring kanamycin resistance.

Table 7.2
Rooting of Potentially Transformed Shoots in Selective Media.

Plasmid	Antibiotic	No. Transferred	No. Rooted	% Rooted
pPCRT-T1	Cm	23	11	50
(cytosol)	Km	55	21	38
pPCRTβcat	Cm	18	2	11
(mit.)	Km	29	12	41
pBIN19	Cm	5	0	0
(Km^r)	Km	38	15	39
GV3101	Cm	8	0	0
(None)	Km	16	0	0

The shoots derived from experiment 1 in Table 7.1 were transferred to MS medium containing chloramphenicol at 10 μg/ml (Cm), or Kanamycin at 100 μg/ml (Km). The number of shoots that were transferred is shown, together with the number (and percentage) that had developed roots after 4 weeks. Shoots had been generated from leaf discs infected with *A. tumefaciens* strains harbouring one or none of the plasmids indicated.

It has already been noted that the growth response to chloramphenicol shown by both resistant and sensitive plant tissue differs depending upon the culture conditions that are used (see section 6.5). This may be due to the K3 medium or bead type culture used for regenerating protoplasts being particularly unsuitable for selection with chloramphenicol, or to growth on solidified MS medium (this work) and solidified Gamborg B5 medium (De Block *et al.* 1984)

Figure 7.3

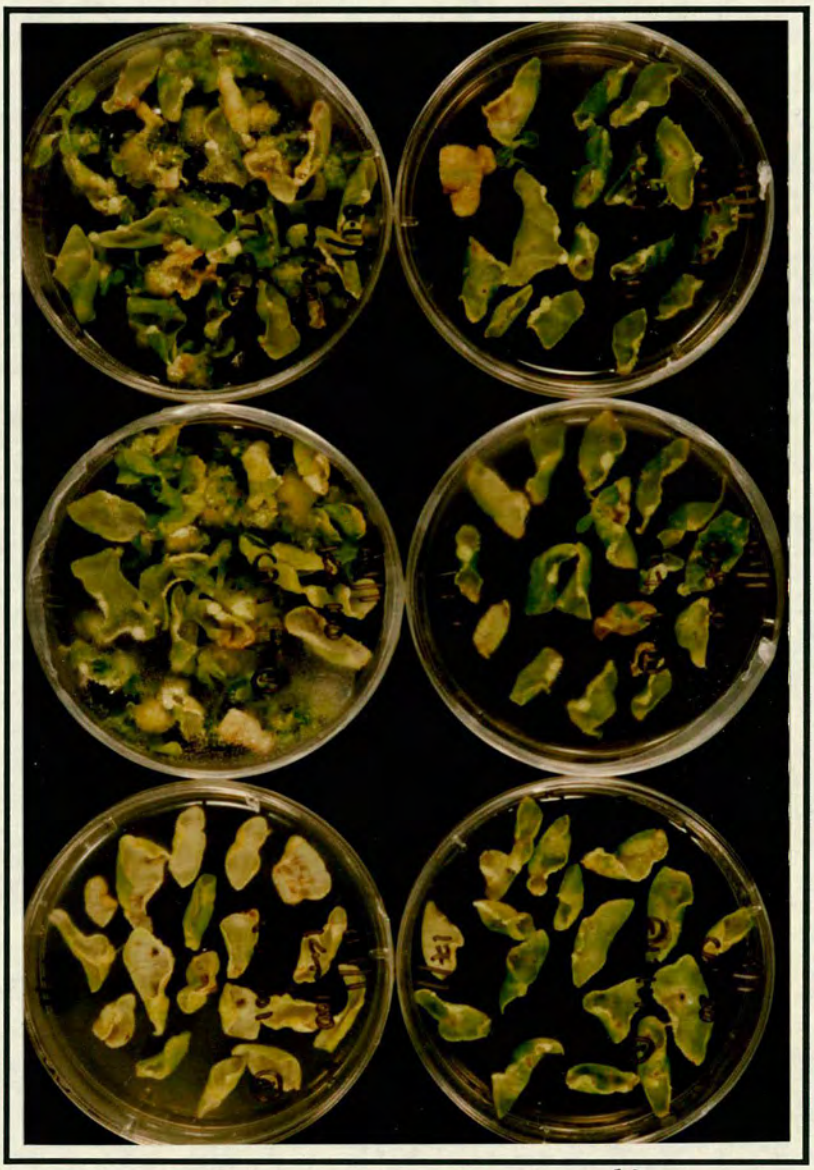
Selection of Chloramphenicol and Kanamycin Resistant Shoots from Leaf Disc Transformation Experiments.

Leaf pieces from sterile *N. tabacum* cv. Petit Havana SR1 were inoculated with *A. tumefaciens* and cultured as described in the text, except that transformed shoots were selected for using chloramphenicol (Cm) at 30 µg/ml or kanamycin (Km) at 100µg/ml. Two Ti-plasmid derivatives were used in this experiment, pPCcatE and pPCcatP. These plasmids are derivatives of the binary vector pPCV706 (Figure 7.1) and are designed to express the Type I and *P. mirabilis* CAT genes from the PL700 promoter of *Solanum tuberosum* (Koncz and Schell 1986, and Dr. C. Bachem personal communication). These plasmids each contain a selectable kanamycin resistance determinant.

Leaf pieces were inoculated with *A. tumefaciens* strain GV3101 MP90RK harbouring one of the two plasmids, or with the *A, tumefaciens* harbouring neither plasmid. After culture for four weeks under selection, the leaf pieces were photographed.

The *A. tumefaciens* strain used to inoculate the leaf pieces on each plate and the antibiotic used to select for transformed shoots are indicated. Shoots were recovered from the leaf pieces only if the infecting bacteria harboured one of the binary vectors.

Figure 7.3


pPCcatE
pPCcatP
GV3101 alone.
Cm
30 µg/ml
Km
100 µg/ml
Cm
30 µg/ml
Km
100 µg/ml

being particularly suitable. The sensitivity to chloramphenicol shown by protoplast derived calli changes during culture in the agarose bead system raising the possibility that the sucrose concentration, which is decreased from 0.4 M to 0.1 M over this period, affects their response to the antibiotic. MS medium also contains about 0.1 M sucrose. Thus, the problems encountered with selection of transformed protoplasts may have resulted from the use of K3 medium, so an alternative protocol involving different conditions and medium (van den Elzen *et al.* 1985) was adopted in the hope that the efficiency of selection with chloramphenicol could be improved. This procedure uses an agarose bead culture system, but with a medium (SII) containing 0.1 M sucrose and mannitol to maintain the required osmolarity.

Protoplasts were isolated from *N. tabacum* cv. Petit Havana SR1, and $7x10^5$ were inoculated in 7 ml of culture medium with $7x10^7$ agrobacteria from a log phase culture of GV3101 MP90RK harbouring pPCRTβcat. Culture conditions were as described by van den Elzen *et al.* (1985), and selection with chloramphenicol and kanamycin was performed exactly as described for the standard PEG-calcium nitrate experiments (Materials and Methods). A similar number of protoplasts were left uninoculated, but cultured in the same way. Each sample was divided between four dishes, and two were selected with chloramphenicol, and two with kanamycin.

The uninoculated protoplasts generally regenerated more rapidly than the others, however kanamycin resistant calli appeared from the latter after five weeks and chloramphenicol resistant calli after six weeks. The transformation frequency was estimated after 11 weeks (Figure 7.4A). About 1000 kanamycin resistant, and 140 chloramphenicol resistant calli were counted on the four plates. If all these were transformants, this is equivalent to a transformation frequency of 3000 and 400 transformants per million protoplasts with kanamycin and chloramphenicol respectively. This transformation frequency is low compared to other reports for this technique when the NPTII gene is selected for using kanamycin (section 7.2.1), so it may still be improved. However in comparison with the procedures used previously, even this represents a dramatic increase of more than 100 fold in the number of chloramphenicol resistant transformants recovered. The recovery of transformants following selection with chloramphenicol relative to selection with kanamycin increased by 5 fold suggesting that the culture conditions and transformation procedure may both have contributed to the increased transformation frequency. Thirty potential transformants were transferred to MS supplemented with chloramphenicol at 10 μg/ml and all continued to grow confirming their resistant phenotype (Figure 7.4B). Two calli were tested for CAT activity, and both demonstrated high levels (Figure 6.7).

Thus the cocultivation procedure seems promising and the experiment

above is being repeated to determine the reproducibility of the improved transformation efficiency that was observed. Cocultivation and the agarose bead culture system have the additional advantage that they are easy to scale up without a corresponding increase in labour. *Agrobacteria* are never in limiting supply, and agarose beads can potentially be cultured together in large numbers for ease of manipulation. Generation of protoplasts is the limiting factor. To regularly generate over 10 million mesophyll protoplasts would require large though not unfeasible numbers of plants; however 20 million protoplasts can be isolated with ease from suspension cultures of *N. tabacum* cv. Xanthi. Such protoplasts are competent for direct DNA transformation (section 4.4.2.1) but do not divide, though protoplasts regenerated from freshly initiated suspension cultures can be regenerated (Uchimaya and Murashige 1974). Therefore a suspension culture has been initiated from *N. tabacum* cv. Petit Havana SR1 as a source of protoplasts for cocultivation experiments. Furthermore, intact suspension culture cells have been shown to be efficiently transformed by *A. tumefaciens* ; about 60 % of microcalli treated are transformed (Draper *et al.* 1988). This provides an even more convenient substrate for transformation.

The potential benefits of such a transformation system are clear, and once established it should provide the best available facility for large scale transformations with the Ti-plasmid derived mitochondrial transformation vectors. Based on the current estimate of the transformation frequency, in 10 experiments with just 10 million protoplasts, 40, 000 potential transformation events may be achieved, which is 20 times greater than was required to achieve yeast mitochondrial transformation using high velocity microprojectiles (Johnston *et al.* 1988, Fox *et al.* 1988). Section 8.5 contains a more extensive discussion of the suitability of the various DNA delivery systems that may be applied to plant mitochondrial transformation.

7.3 A CAT Gene Designed for Plant Mitochondria.

Expression of the CAT gene exclusively in mitochondria is central to the strategy adopted for development of a mitochondrial transformation system. The required specificity of expression has been attempted by constructing vectors containing mitochondrial gene expression signals to express the CAT gene from *Proteus mirabilis* . The nuclear and cytosolic expression system seems not to synthesise sufficient CAT from these sequences to allow nuclear transformants to survive the selection system and hinder the identification of mitochondrial transformants (see sections 4.4 and 6.6). However, the possibility remains that when larger numbers of protoplasts are transformed, integration of *cat* sequences

Figure 7.4

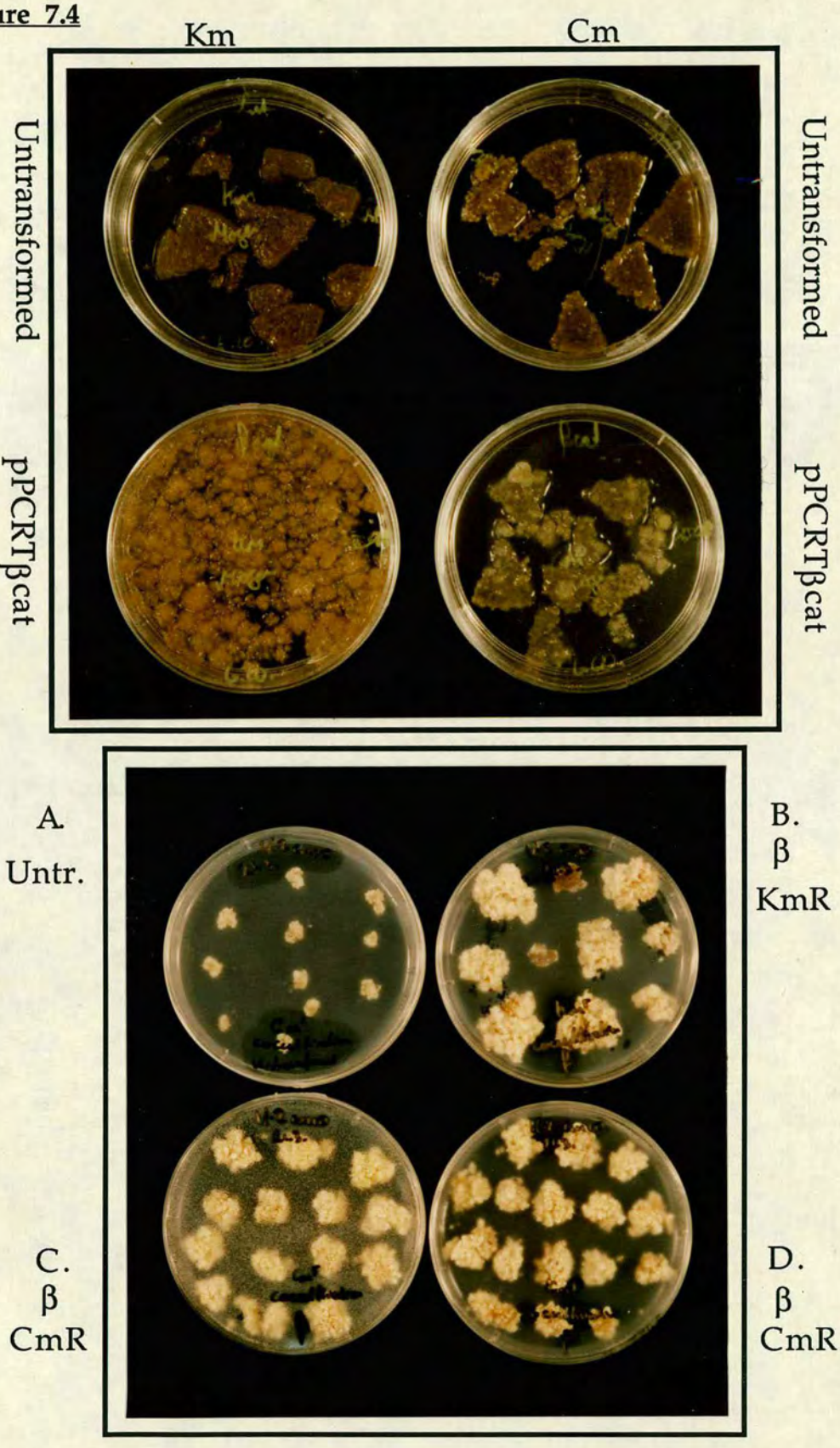
Selection of Chloramphenicol and Kanamycin Resistant Calli Following Cocultivation of Protoplasts with *A. tumefaciens*.

A. Conditions for cocultivation and selection are described in the text. Mesophyll protoplasts isolated from *N. tabacum* cv. Petit Havana SR1 were infected with *A. tumefaciens* strain GV3101 MP90RK harbouring pPCRTβcat (Figure 7.1), or were left uninoculated. The cultures were selected for either chloramphenicol resistant calli (Cm) or kanamycin resistant calli (Km) and were photographed ten weeks after transformation.

B. 30 of the apparently chloramphenicol resistant calli (CmR) recovered from the cocultivation experiment described above were transferred to petri dishes containing Murashige and Skoog medium supplemented with chloramphenicol at 10 μg/ml. In addition, ten kanamycin resistant transformants (KmR) and eleven untransformed calli (Untr.) were transferred onto similar medium. Calli were photographed after approximately four weeks.

All apparently chloramphenicol resistant calli continued to grow under these conditions, whereas the untransformed calli showed no significant growth. Eight of the ten kanamycin resistant calli showed resistance to chloramphenicol on this medium, showing that *preβcat* can confer chloramphenicol resistance even if this is not directly selected for. The inactivation of a cotransferred nonselected marker in a significant percentage of transformants has been widely reported (for example Velten and Schell 1985, Czernilofsky 1986a).

Figure 7.4


Km
Cm
Untransformed
pPCRTβcat
Untransformed
pPCRTβcat
A.
Untr.
B.
β
KmR
C.
β
CmR
D.
β
CmR

will occur by chance near nuclear gene expression signals, resulting in expression of *cat* at a frequency which could impede identification of a mitochondrial transformant. In addition, when *A. tumefaciens* is used to mediate transformation, the potential exists that a low level of expression of *cat* in the bacteria may lead to cross protection of nontransformed plant cells.

To provide absolute specificity for expression of *cat* in plant mitochondria it was decided to exploit the difference between the genetic code used in higher plant mitochondria and the standard genetic code used in the expression of nuclear and bacterial genes (section 3.1.2). If the tryptophan codons (TGG) of the selectable marker are converted to CGG which is thought to encode tryptophan in the plant mitochondrial genetic code, then even if the gene is expressed from the nucleus or in *Agrobacterium* the protein product will probably not retain enzyme activity. Such a strategy seemed possible with CAT as there are several highly conserved tryptophan residues in the variants whose sequence has been deduced to date (Figure 3.1).

Therefore, site specific mutagenesis was performed in collaboration with Dr. I.A. Murray, University of Leicester, on a CAT gene from *E. coli* that encodes a Type III variant of the enzyme (Figure 3.1, Murray *et al.* 1986). This enzyme has the highest specific activity of any CAT variant analysed to date (Kleanthous and Shaw 1984). Two of the arginine residues of the Type III gene are normally encoded by CGG codons, so initially these were converted to CGU, which was found to be the preferred codon for arginine in those plant mitochondrial genes whose sequence had been determined (Table 7.3).

Table 7.3
Arginine Codon Usage in Several Plant Mitochondrial Genes.

Plant Species	Gene	Codon CGU	CGC	CGA	AGA	AGG
Maize	*atp9*			1		
	atpA	6	7	8	8	2
	coxI	6	1	4	4	
	coxII	4	1	2	1	1
	cob	4	1	3	2	2
Oenothera	*coxII*	3	1	1	1	1
	cob	6	1	2	2	2
Pea	*coxII*	2	1	1	1	1
Rice	*coxII*	3	1	2	1	1
Sum		34	14	24	20	10

As expected, introduction of these two mutations did not affect the activity of CAT synthesised in *E. coli*. (I.A. Murray, personal communication). The gene encodes three tryptophan (Trp) residues, all of which were mutated to CGG. Surprisingly, conversion of the codon for Trp 11 to CGG had no detectable effect on enzyme activity expressed in *E. coli* despite this residue being conserved in five of the six known variants of CAT. The corresponding codon substitution at Trp 146 reduced activity to 15 %, and when in addition the codon for Trp 80 was converted to CGG, activity could no longer be detected in *E. coli* (I.A.Murray, personal communication). This last mutation introduces a recognition site for *Rsa*I, to allow the mutant and wildtype genes to be easily differentiated.

The crystal structure of Type III CAT has been published (Leslie *et al.* 1988). It forms a homotrimer with the active site composed of residues from two subunits. Trp 11 is located in a region of random coil that precedes the first contact of the polypeptide chain with the adjacent subunit. It may be that this region is hydrophilic or sufficiently exposed to the solvent to accommodate arginine at this position without disrupting the active site which is nearby; the arginine residue at position 13 is involved in binding chloramphenicol. Trp 80 is located in a random coil between two β-sheets, and appears neither to be involved in the active site nor embedded within the core of the protein, so the reason for inactivation by arginine at this position is not clear. Trp 146 however is located in a hydrophobic tunnel which binds the pantothene arm of acetyl coenzyme A where it forms, via two ordered water molecules, one of eight hydrogen bonds to the cofactor.

Construction of Plasmids Containing the Mutated Type III CAT Gene.

A Type III CAT gene containing all five mutations described above was constructed in collaboration with Dr. I. Murray in a 1040 bp *Bam*HI to *Hin*dIII fragment in the M13 vector mp18. The subcloning strategy that is being used to generate mitochondrial transformation vectors with this mutated gene is described below. Initially the *Bam*HI to *Hin*dIII fragment was removed with the appropriate restriction endonucleases, and ligated into the equivalent sites of pUC19 to produce pI-500 (Figure 7.5). The start of the coding sequence is 150 bp 3' to the *Bam*HI site, and a cleavage site for *Cla*I precedes the initiation codon by 5 bp (Figure 7.5). These two sites are unique in pI-500 so the sequence between them was removed by digestion with the respective enzymes, after which the termini were treated with the Klenow fragment of *E. coli* DNA polymerase I to generate blunt ends, and the vector fragment was religated to produce pI-504. The fidelity of this process was assessed by checking for the *Bam*HI site that should be

regenerated upon ligation (in similar fashion to that shown in Figure 3.11). This site is positioned at exactly the same distance from the initiation codon of the Type III CAT gene as the *Bam*HI site upstream of the *P. mirabilis* CAT gene in all the vectors constructed previously with the COXI gene promoter region. Thus, fusion of the Type III CAT gene to the COXI sequence at the *Bam*HI site will create a translational fusion between COXI and the Type III CAT which is similar to the previous fusion between COXI and *P. mirabilis* CAT, with only a single amino acid will differing between the two fusions (Figure 7.6).

The N-terminus of the Type III gene is exposed on the surface of the protein away from both the active site and the contact points between the subunits of the homotrimer. However, this region is five residues shorter than the corresponding portion of the Type I and *P. mirabilis* CAT variants, so although the latter two tolerate N-terminal fusions, this cannot be assumed for Type III CAT, and will be determined as for the previous fusion.

The promoter regions of the N and S type COXI genes were isolated from pUPS92J and pSCOX92O as *Eco*RI to *Bam*HI fragments, (see Figure 3.9) , and inserted between the equivalent sites of pI-504 (Figure 7.5) to produce pJ504 and pS504. The *P. mirabilis* CAT gene in the transformation vectors constructed previously is flanked by *Bam*HI sites (Figure 3.9), so to facilitate its easy replacement by the mutated Type III variant, a second *Bam*HI site was inserted downstream of the latter gene. This was done by transferring the *Eco*RI to *Hpa*I fragment of pJ504 and pS504 that contains the chimaeric Type III gene into the *Eco*RI and *Sma*I sites of pK18 (Figure 7.5). Plasmid pK18 has a *Bam*HI site adjacent to its *Sma*I site which will allow the mutated gene to be isolated on a *Bam*HI fragment. The intermediate plasmids described above have been constructed (not shown), but have not been used in transformation experiments; the *Bam*HI fragment containing the Type III gene is being used to replace the *P. mirabilis* gene in the pUC9 and Ti-plasmid based transformation vectors, and these will be incorporated into the future transformation programme (Figure 7.2).

Figure 7.5

Scheme for Construction of Mitochondrial Transformation Vectors Containing the Mutated Type III CAT Gene.

Plasmid pI-500 shown at the top of the figure comprises a 1 kb *Bam*HI (B) to *Hin*dIII (H) fragment containing the mutated form of the Type III CAT gene (shaded box, arrow shows direction of transcription) inserted in the polylinker of pUC19 (*Eco*RI (E) to *Hin*dIII). This plasmid was digested at its unique *Bam*HI and *Cla*I (C) recognition sites and religated to remove the internal sequence and introduce a *Bam*HI site upstream of the initiation codon of the CAT gene (plasmid pI-504).

The *Eco*RI to *Bam*HI fragments from pUPS92J and pSCOX920 that contain the N and S type maize COXI gene promoter region and N-terminal coding sequence were inserted at the equivalent sites in pI-504 as shown. The N terminal sequence of the resulting fusions to the Type III CAT will be identical to the fusions to the *P. mirabilis* CAT that were constructed previously, except for one residue (Figure 7.6). The pI-504 derivatives containing the N and S type COXI sequences were called pJ504 and pS504 respectively.

Several restriction endonuclease recognition sites including a *Bam*HI site were introduced at the 3' end of the mutated Type III CAT gene. This *Bam*HI site allows the Type III gene to be isolated as a *Bam*HI fragment which will facilitate its insertion into many of the mitochondrial transformation vectors constructed previously by replacement of the *P. mirabilis* CAT gene which also can be isolated as a *Bam*HI fragment. Plasmids pJ504 and pS504 were digested with *Eco*RI and *Hpa*I (Hp) and the fragment containing the chimaeric CAT gene was ligated into the *Eco*RI and *Sma*I (Sm) sites in the polylinker of pK18 (identical to that of pUC18/19) to produce plasmids pKJ500 and pKS500, of which the former is shown at the bottom of the figure. Xb, Sl, P, and Sp indicate recognition sites for *Xba*I, *Sal*I, *Pst*I, and *Sph*I respectively.

Figure 7.5

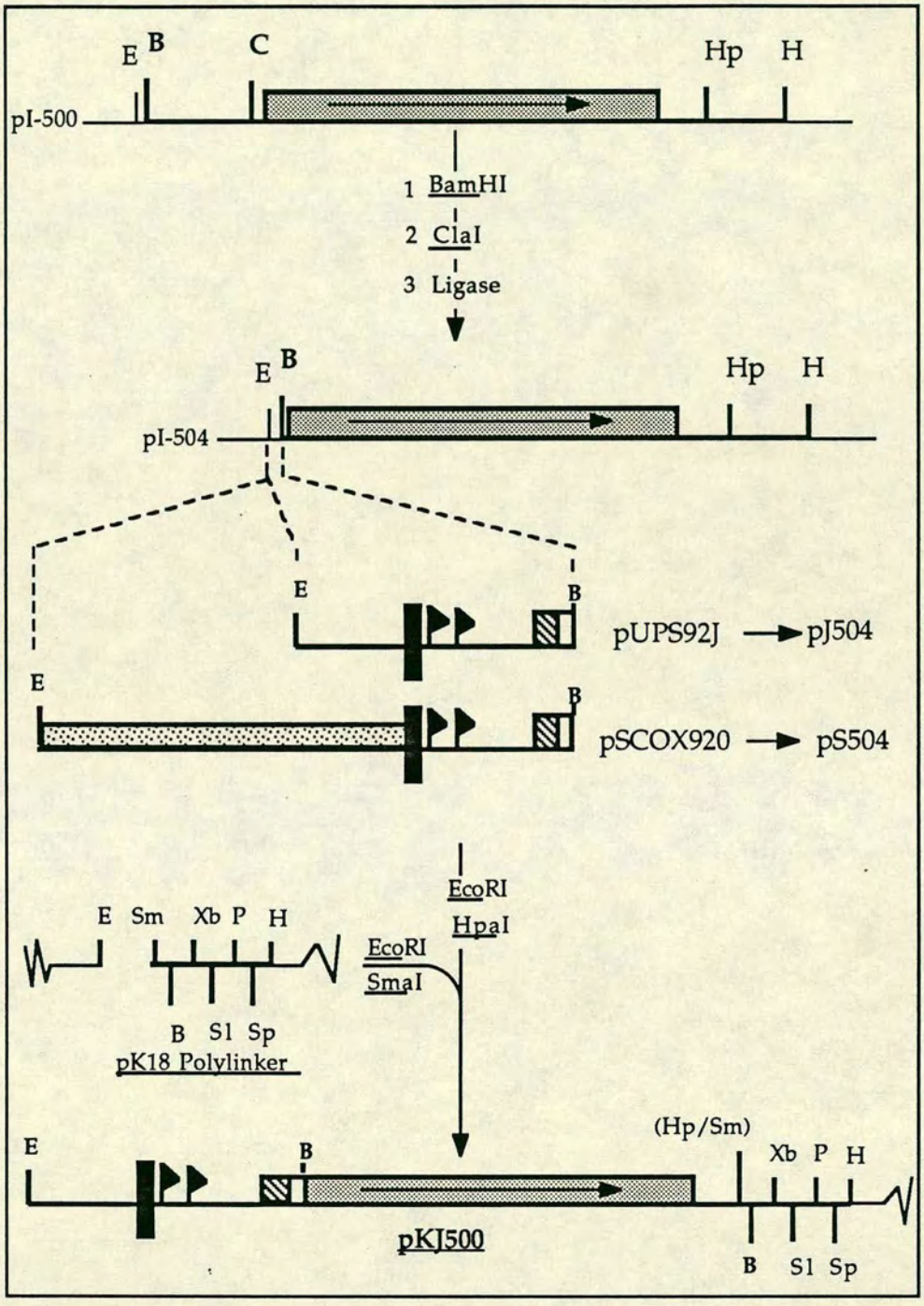

E B C Hp H
pI-500
1 BamHI
2 ClaI
3 Ligase
E B Hp H
pI-504
E B
pUPS92J → pJ504
E B
pSCOX920 → pS504
EcoRI
HpaI
E Sm Xb P H
EcoRI
SmaI
B S1 Sp
pK18 Polylinker
(Hp/Sm)
E B Xb P H
pKJ500
B S1 Sp

Figure 7.6

Comparison of the N-Terminal Fusions Between COXI and either the Type III and *P. mirabilis* Variants of CAT.

CAT Gene Source	Sequence of the N-Terminal Extension
	Met Thr Asn Leu Val Gly Asp Pro Ile Met...
P.mirabilis	5' ATG ACA AAT CTG GTC GGG GAT CCT AAT ATG...
Type III	5' ATG ACA AAT CTG GTC GGG GAT CCG ATT ATG...
	Met Thr Asn Leu Val Gly Asp Pro **Asn** Met...
	1. 2.

The sequence of the N terminal fusions to the two CAT variants are shown in standard three letter notation directly above or below the DNA sequence that encodes them. The two residues that differ are in bold face, and the *Bam* HI sites that were used in the construction are underlined. The initiation codons that initiate translation of the extended protein and of the normal CAT protein are numbered 1 and 2 respectively.

CHAPTER 8.

SUMMARY AND DISCUSSION.

8.1 Summary

Eukaryotic genomes are complex, being divided between as many as three different intracellular compartments. In all cases studied so far, the majority of the cellular DNA and coding capacity is in the nucleus; however mitochondria also contain DNA which has been shown to encode a small number of active genes, and in addition, plant cells contain a third small genome in the chloroplasts. Mitochondria and chloroplasts have fundamental roles in the provision and conversion of energy in plant cells. Reduced cofactors generated by glycolysis in the cytosol and by operation of the TCA cycle in the mitochondrial matrix are oxidised by oligomeric protein complexes in the inner mitochondrial membrane. Their oxidation is concomitant with translocation of protons out of the matrix, thus establishing a proton gradient across the membrane. The return of protons to the matrix is coupled to ATP synthesis by the F1-F0 ATP synthase. In plants, mitochondrial function is required for many developmental processes from germination through to flowering; an insight into the importance of proper mitochondrial function is provided by the NCS and commercially important CMS phenotypes that appear to result from mutations in the mitochondrial genome.

The 'organellar' genomes, those of chloroplasts and mitochondria, encode a largely conserved group of RNAs and proteins, which are synthesised and appear to function entirely within their respective organelles. Mitochondria are estimated to be able to synthesise between 13 and 30 polypeptides (depending on the organism studied), which constitute only 5 to 10 % of the total protein complement of the organelle; the remaining proteins are thought to be products of nuclear genes. As the gene products encoded within the mtDNA are required for proper mitochondrial function, a knowledge of mitochondrial gene expression is necessary for a complete understanding of the biology of these organelles.

Gene structure and expression in prokaryotes and latterly in eukaryotes has become increasingly understood as the development of *in vitro* techniques for manipulation of nucleic acids has advanced. The key to much of this progress has been the ability to isolate specific nucleic acid sequences, to determine their structure and abundance, and to correlate changes in these parameters with particular physiological processes or with defined mutant phenotypes. However, for the development of functional explanations of gene action, perhaps the most rewarding approach has been to introduce specific alterations into an isolated DNA sequence, and then to determine their effect upon the function of the sequence in a suitable *in vitro* assay, or preferably *in vivo* following transformation with the modified DNA sequence. Presently, only the former techniques are available for analysis of plant mtDNA, and informative mutations are scarce. This confines analysis to detailed description of the organisation and expression of the genome, but provides no means to test the functional significance of structures or of variations in gene expression which are observed.

A transformation system for plant mitochondria would therefore be of value in the investigation of mitochondria and mtDNA, and may eventually allow these organelles to be beneficially modified. A variety of techniques for delivery of DNA into plant cells are now available. The fate of DNA transferred into plant cells is poorly understood raising the possibility that some fraction of transforming DNA enters the mitochondria. At the inception of the work described in the thesis, it had been shown by others that a portion of the transforming DNA becomes integrated into the nuclear genome of plant cells, and that such cells could be recovered if the transforming DNA contained chimaeric nuclear genes that conferred a selectable antibiotic resistant phenotype to nuclear transformants. Clues to the sequences required for expression of plant mitochondrial genes were also emerging. In an attempt to recover plant cells that contain transforming DNA in their mitochondria a strategy was devised based upon the construction of selectable antibiotic resistance genes that would function in only the mitochondria.

Summary of the Results, Chapters 3 to 7.

The strategy devised for recovering a mitochondrial transformant can be summarised as follows:

I. The construction of mitochondrial transformation vectors. These comprise a selectable marker gene coupled to various sequences derived

from plant mtDNA to promote expression and maintenance of the marker specifically in the mitochondria of plant cells. Expression of the marker gene in mitochondria was intended to confer resistance to an inhibitor of plant cell growth, providing a selectable phenotype.

II. the optimisation of culture conditions for selection of transformants after transformation of plant cells with the mitochondrial transformation vectors.

III. the use of several DNA delivery systems to transform plant cells in an attempt to find the one most suited to mitochondrial transformation.

IV. once the above have been established, it is envisaged that foreign genes or mitochondrial genes could be included in the transformation vectors, delivered to plant mitochondria, and plants containing these sequences in their mitochondria recovered by selection for the cotransferred marker gene.

In this thesis I have addressed the first three of these points. Chloramphenicol was chosen as the most suitable of the antibiotics that had previously been successfully employed as selective agents for recovery of plant nuclear transformants. The alternative antibiotics, kanamycin, hygromycin and methotrexate were discounted owing to the weak resistance conferred by the relevant selectable marker, or to doubts about their site of action and about the permeability of the mitochondrial membranes to the antibiotic. The chloramphenicol acetyltransferase (CAT) gene (*cat*) from *P. mirabilis* was chosen as the selectable marker for inclusion in the transformation vectors. This variant of the CAT gene is similar to the gene from Tn*9* that had been used previously to confer chloramphenicol resistance to nuclear plant transformants, but has the advantage that it contains no CGG codons that may be mistranslated in plant mitochondria. To facilitate expression of *cat* in plant mitochondria, it was provided with the putative promoter sequences and untranslated mRNA leader sequences of the *Petunia hybrida* ATP9-1 gene or the maize COXI gene. In an attempt to provide all necessary translation signals in the latter construct, CAT can be synthesised as a fusion to the N terminus of COXI. The integrity of the fused open reading frame was confirmed by nucleotide sequence determination, and genetic evidence that the fusion protein retains enzyme activity was obtained.

The mitochondrial transformation vectors were shown to confer weak chloramphenicol resistance to bacteria suggesting a low level of expression. They failed to express the CAT gene at detectable levels in tobacco callus after stable nuclear transformation or in transient expression analysis in tobacco protoplasts; if these vectors are active in mitochondria, it appears that the required specificity

of expression has been achieved by the use of the mitochondrial gene expression signals.

In a second approach, I attempted to provide additional specificity for selection of mitochondrial transformants by modifying the codon usage of a bacterial gene encoding the Type III variant of CAT. The result of these modifications is that enzymatically active molecules will be synthesised only if the CGG codons are translated as tryptophan as is thought to be the case in plant mitochondria. A derivative of the Type III CAT gene in which the three TGG codons (encoding the three tryptophan residues) were converted to CGG (encoding arginine in the standard genetic code) was unable to express CAT activity in *E. coli*. Although each of these tryptophan residues is highly conserved amongst the six known isolates of the CAT gene, replacement of tryptophan by arginine at one of these positions appeared to have no effect upon the enzyme activity expressed in *E. coli*, whilst similar replacements at the two other positions severely reduce this activity. The case for assigning CGG as a codon for tryptophan in plant mitochondria rests upon the observation that CGG codons in plant mitochondrial genes align with highly conserved tryptophan residues in homologous mitochondrial proteins. However, some CGG codons have been found at positions that align with arginine residues (Table 3.4). The variable tolerance of CAT to substitution of a conserved tryptophan by arginine illustrates the difficulty in assessing the case for assigning CGG as a tryptophan codon in plant mitochondria on the basis of sequence comparisons (section 3.1.2). Whatever its true specificity in plant mitochondria, and perhaps weight of numbers favours tryptophan, the observation that CAT can apparently tolerate arginine in place of a conserved tryptophan makes it less anomalous that CGG codons in plant mitochondrial genes align with conserved residues of both amino acids.

To maintain the transforming DNA in mitochondria, restriction endonuclease fragments derived from cloned portions of tobacco and *Petunia* mitochondrial DNA were incorporated into the vectors. These sequences have the potential for homologous recombination with the mitochondrial genome of the host cell. A potential transcription terminator was also included in some of these constructs.

A selection procedure has been developed to allow the CAT gene to be used as a reliable selectable marker for transformation. Chimaeric nuclear genes that express *cat* have been used to obtain chloramphenicol resistant nuclear transformants following transformation of tobacco cells using protoplasts and leaf discs, the two systems currently used most frequently. Both direct DNA transfer and *A. tumefaciens* were used to generate the transformants. These transformation and selection techniques were also used successfully with a chimaeric CAT gene whose product had been shown to be targeted to tobacco

mitochondria owing to the incorporation of a mitochondrial targeting sequence from the β subunit of the F1 F0 ATP synthase (Boutry *et al.* 1987). Ti plasmid derivatives containing the selectable markers for mitochondrial transformation were constructed for use in cocultivation experiments. This technique was found to provide the highest yield of transformants, making it the established method of choice.

Initial direct DNA transfer experiments have been performed with the mitochondrial transformation vectors, but low transformation frequencies prevented detailed conclusions to be drawn. No evidence for mitochondrial transformation was obtained. The chloramphenicol resistance of the potential transformants was low and usually not sustained during subculture. Elevated CAT activity was not detected in these calli or in those which continued to express weak resistance.

The implications of these preliminary experiments for the continued use of this mitochondrial transformation strategy, for its modification, and for development of alternative strategies during the remainder of this project are discussed in turn below.

8.2 Chloramphenicol Acetyltransferase as a Selectable Marker.

Successful use of *cat* for selecting transformed plant cells was first reported by De Block *et al.* (1984). They were able to select nuclear transformants easily only after transfer of microcalli to solid medium containing 5 μg/ml chloramphenicol. They were unable to achieve a reliable or discriminatory selection system using K3 medium and the agarose bead culture system: selection could not be performed above 10 μg/ml chloramphenicol; transformants were indistinct and had to be screened on solid medium; this could be done only after at least 3 weeks growth in the absence of chloramphenicol, and resistant calli could not be maintained on chloramphenicol containing medium for more than 2 months. Consequently, use of chloramphenicol has been reported only once since then (Pietrzak *et al.* 1986), and Weising *et al.* (1988) do not consider the CAT gene to be a suitable selectable marker in their recent compilation of chimaeric genes used for expression in plant cells.

The range of techniques and culture conditions with which *cat* and chloramphenicol can be used has been extended by the results reported in Chapters 6 and 7 to include the leaf disc and agarose bead culture systems. The chloramphenicol selection system described in this thesis is:

1. Reliable. A false positive has not been obtained. The reliability stems from increased discrimination between resistant and sensitive

phenotypes. In addition the resistant phenotype can be easily confirmed by subsequent screening on MS medium supplemented with chloramphenicol at concentrations between 10 μg/ml and 60μg/ml.

2. Convenient. A basic three stage selection system was devised and has been successfully used with two different tobacco protoplast culture systems and the leaf disc transformation system following transformation with a variety of CAT expressing constructs.

If there is a problem with this selection system it is that selection may be too stringent as chloramphenicol resistant calli were recovered at a frequency only 10 to 20 % of that for kanamycin resistant calli generated by transformation with chimaeric NPTII genes. Although the frequency of recovery of chloramphenicol resistant calli could probably be improved, the current system is sufficient for most nuclear transformation purposes. In most experiments, delivery of a particular non-selectable gene construct to the plant nucleus requires the recovery of about 10 transformed plants as a representative sample (different transformants vary in their level and pattern of expression of transferred sequences Dean *et al.* 1988); this number could easily be recovered from either naked DNA or *A. tumefaciens* mediated transformation experiments of the type reported in this thesis.

There are several possible explanations for the observed difference in the number of chloramphenicol and kanamycin resistant transformants recovered from similar transformation experiments:

1. It has been widely documented that genes transferred into plant nuclei are expressed at levels that vary between individual transformants. This variability has been attributed to the position of the transferred genes in the chromosome, to their copy number and to differential methylation (Dean *et al.* 1988, reviewed in Weising *et al.* 1988). The transcription and translation signals used to express *cat* may have been less efficient than those used to express *nptII* so fewer transformants may have synthesised sufficient CAT to afford resistance. It is doubtful that this is the sole reason; the promoters used to transcribe *cat* in pRT-T1 and pCAP212 (35S and T-DNA 1' promoter respectively) are both reported to be stronger than the nopaline synthase promoter used to transcribe *nptII* in pLGV1103 (Sanders *et al.* 1987, Harpster *et al.* 1988). Secondly, the efficiency with which chloramphenicol resistant calli were recovered following transformation with pCAP212 and pRT-T1 was similar though their expression signals were different (Table 6.1); likewise, both *nptII* expressing plasmids, pCAP212 and pLGV1103, generated similar numbers of transformants though the promoters were different. A quantitative estimation of the CAT activity in pCAP212 transformants selected with either chloramphenicol or kanamycin would reveal whether or not chloramphenicol selects for a subpopulation of calli in which expression of the CAT gene is relatively high. Although such analyses conducted

in this work, for example Figure 6.9B, appear to show greater CAT activity in calli selected with chloramphenicol, these assays were not strictly quantitative, and thus provide no clear support for selection of calli that contain high levels of CAT activity. In addition, to show that such a selection process accounted for the difference between the recovery of chloramphenicol and kanamycin resistant calli, it would be necessary to show that a similar selection for calli containing the highest levels of NPTII activity did not operate during selection of transformants with kanamycin.

2. It is possible that *cat* or its regulatory sequences are more prone to inactivation, for example by rearrangement or methylation.

3. If expression of *cat* is not unusually low or susceptible to interference, the enzyme may not be sufficiently active to effectively reduce the intracellular chloramphenicol concentration, particularly if diffusion into the cells is rapid. NPTII may have a higher specific activity, or kanamycin may be less permeable.

4. The supply of acetyl coenzyme A required to inactivate the chloramphenicol may be limiting. Alternatively, CAT may compete poorly for the available cofactor. The apparent K_m of CAT for acetyl coenzyme A has been estimated at about 0.08 mM (Charles *et al.* 1985a) compared to that for a coenzyme A transport activity in plant mitochondrial membranes of 0.2 mM (Neuberger *et al.* 1984), although this latter value may be atypical of other other enzymes, for example citrate synthase which has a much lower K_m (Douce 1985). Thus it remains possible that CAT competes poorly for intracellular acetyl coenzyme A, or that efficient acetylation of chloramphenicol limits the availability of the cofactor for other processes. If ^{14}C labelled chloramphenicol was added to the culture medium, the rate of its acetylation in the medium and in transformed and wild-type callus could be followed, and this may reveal if any of the above possibilities were relevant *in vivo*.

5. The acetylated forms of chloramphenicol may still inhibit organellar translation, or they may have some other toxic effect distinct from that of unmodified chloramphenicol.

6. The effects of chloramphenicol on processes other than translation (section 3.2), for example sulphate ion uptake, may not be moderated by its acetylation. Replacing the chloramphenicol in the culture medium with its acetylated forms may assist in the evaluation of the latter possibilities.

This latter possibility may explain in part the differing responses to chloramphenicol that were observed when wild-type and transformed tissue was cultured under different conditions (sections 6.3.2 and 6.5). The differences may have been mediated in part by the effect of growth conditions on many of the metabolic processes described above, for example, ion concentrations in the media may be of importance if their uptake is affected by chloramphenicol. The most

discriminatory selection conditions were obtained using agar solidified MS medium. As microcalli were transferred to this medium following regeneration from protoplasts in protoplast culture media the different responses may have been due to the age or organisation of the tissue rather than the composition of the media. Two observations argue against this. Firstly, resistant calli mainain their characteristic response to chloramphenicol in each medium if they are transferred to MS immediately after they emerge or are left several weeks before transfer. Secondly, in preliminary experiments in which protoplasts were regenerated directly on osmotically buffered MS medium, their response to chloramphenicol was found to be similar to that of callus (not shown). Although the specific ionic concentrations of the different culture media may affect the sensitivity of plant tissue to chloramphenicol, this would be laborious to analyse systematically.

MS medium containing chloramphenicol has been found to provide an effective low stringency screen for chloramphenicol resistance (section 6.5), so attempts are underway to use this for routine large scale selection of regenerating protoplasts in the hope that the level of resistance required to survive selection will be lowered. This is of general value, but may also be of particular relevance to selection of mitochondrial transformants.

8.3 Evaluation of the Strategy Adopted for Recovery of Mitochondrial Transformants.

Chapters 1 and 3 contain detailed discussions of the process of mitochondrial gene expression in higher plants. Based on the information available in 1985 and 1986, chimaeric, potentially selectable, CAT genes were designed for inclusion in mitochondrial transformation vectors. These vectors contained mtDNA sequences coupled to the CAT gene coding sequence to facilitate its expression in plant mitochondria. Discussion of the suitability of these constructs would only reiterate the discussion in the earlier chapters, and like these, it would not be conclusive. In practice, alternative strategies for expression of *cat* could have been adopted, and it is certain that many more alternative constructs could have been designed than could have been assembled; already more constructs have been assembled than could be tested thoroughly by transformation. Finally the choice of vectors for transformation studies must comprise a small number of representative alternatives; the constructs described in the preceding chapters are such a group.

The strategy of attempting to promote recombination between the transforming DNA and the resident mitochondrial genome seems to have been

vindicated by recent results with organelle transformation. Boynton *et al.* (1988) have shown that transforming DNA is probably integrated into the chloroplast genome of *C. reinhardtii* by homologous recombination, and Johnston *et al.* (1988) reached similar conclusions after transformation of mitochondria in *S. cerevisiae*. Many of these events seem to have resulted from double crossovers leading to exchange of the resident and plasmid borne sequences, however Blowers *et al.* (1989) found that additional DNA can be accommodated in the chloroplast genome without selection for its presence. It had previously been thought that such additional DNA sequence would be unstable in the highly conserved chloroplast genome. The transforming plasmid was initially maintained independently of the chromosome for a few generations and may have been replicating though it was not known to possess a plastid origin of replication. Similarly, ρ0 mutants of yeast that lack mitochondrial DNA were able to stably maintain the transforming plasmid as repeated concatamers (Fox *et al.* 1988). This type of amplification process is a feature of mitochondrial mutants that retain only a small part of the normal mitochondrial genome following large deletions. This small portion of the genome becomes amplified to restore the normal mtDNA complement. Transformation of ρ0 yeast strains is thought to mimic this event (Fox *et al.* 1988). Such maintenance of the transforming plasmid is not observed in wild type strains however, and its relevance to plant mitochondrial transformation is doubtful.

The maintenance strategy described in this thesis relies upon homologous recombination between the transformation vectors and the tobacco mitochondrial genome in the region of the ATP9 gene and the S13 and NAD1 homologous reading frames. The unknown factor in the strategy is the potential effect of such a recombination event upon the expression of the endogenous NAD1 reading frame and thus on mitochondrial function. This is difficult to assess as the mode of expression of *nadI* remains unknown, but this has been discussed in Chapter 5. If *nadI* is functional in tobacco, it probably encodes subunit 1 of the NADH ubiquinone oxidoreductase complex (Complex 1). Inhibition of this complex with rotenone disrupts mitochondrial activity in animal mitochondria and is therefore toxic (Ravanel *et al.* 1984). Plant cells have several alternative NADH dehydrogenases (section 1.1), two of which are coupled to two sites of ATP production, so it is theoretically possible that even if the function of Complex 1 was impaired, the integrated foreign sequences could be maintained in a sufficient number of mitochondrial genomes to allow chloramphenicol resistance to be expressed.

The major potential constraints to the success of the mitochondrial transformation strategy that remain to be considered are the frequency at which mitochondrial transformation can be expected to occur, and the level of antibiotic resistance that such transformants can be expected to show. The former is

probably dependent upon the method of transformation that is employed, and receives attention in a later section. Although an experimental approach will be required to finally determine the level of chloramphenicol resistance that mitochondrial transformants could achieve, a consideration of some relevant published data and of results reported in this thesis may be of assistance in its estimation.

Nuclear transformation experiments were performed with plasmid pRTpreβcat that encodes a chimaeric CAT (preβcat) which is targeted to plant mitochondria owing to its fusion to the N-terminal targeting sequence of ATPB from *N. tabacum* (Boutry *et al.* 1987). However, when this plasmid was used for transformation fewer transformants were recovered than when pRT-T1 was used; the latter plasmid encodes a wild-type CAT which remains in the cytosol (Table 6.1, Tables 7.1 and 7.2). Tissue expressing preβcat also appeared to be less resistant to chloramphenicol (shoots were slower to root on chloramphenicol and callus growth is markedly slowed by as little as 20 μg chloramphenicol/ml). This suggests that per molecule of CAT that is synthesised, mitochondrial transformants may be less resistant to chloramphenicol than nuclear transformants. However, transformants expressing the mitochondrial form of CAT have been recovered, so if mitochondrial transformants expressed CAT at a level similar to these transformants, it is probable that they too could be recovered. The amount of CAT accumulated in the chloramphenicol resistant nuclear transformants has not been determined, and the amount of CAT that would be accumulated in mitochondrial transformants depends upon the rate of transcription of the chimaeric gene, the rate of translation of the mRNA, and the stability of the protein. Values for these parameters are not available. Despite this, the following considerations suggest that it may be possible for CAT to accumulate in mitochondrial transformants at levels similar to those in the nuclear transformants considered above.

The efficiency with which the CAT genes are expressed in the chloramphenicol resistant nuclear transformants is not known, however it is potentially relatively high as the 35S promoter from which they are transcribed is amongst the strongest that have been analysed: it is stronger than any T-DNA promoter studied (Sanders *et al.* 1987, Harpster *et al.* 1988); when a cDNA clone of EPSP synthase was fused to the 35S promoter it was expressed at levels between 20 and 40 fold higher than the wild type gene (Shah *et al.* 1986); levels of expression of a bacterial chitinase gene obtained in tobacco cells using the 35S promoter have been matched only by those obtained in leaves using a promoter from a RUBP carboxylase small subunit gene, and only after optimising the mRNA sequence for efficient translation (Jones *et al.* 1988). This last report emphasises the importance of translational efficiency in determining the final level of expression of chimaeric genes. It is not known how efficiently the mRNA transcribed from

the nuclear CAT genes is translated, but the highest published estimates for accumulation of chimaeric gene products in transformed plants range from 0.1 % of soluble cellular protein for expression of the coat protein of Tobacco Mosaic Virus to 0.4 % for a bacterial chitinase, and 0.8 % for the coat protein of Alfalfa Mosaic Virus (Powell-Abel *et al.* 1986, Jones *et al.* 1988, and Tumer *et al.* 1987 respectively), and in each case the 35S promoter was used. From these figures it can be calculated that in the most efficient cases yet reported chimaeric gene products accumulate to between 0.04 and 0.3 nmoles/mg soluble cellular protein, providing an upper limit for estimates of the amount of CAT that is likely to be accumulated in the chloramphenicol resistant nuclear transformants. By comparison, spectrophotometric measurements of cytochromes led Douce (1985) to estimate that the mitochondria of cultured sycamore cells accumulate 0.026 nmoles of the cytochrome$_{aa3}$ per milligram total cellular protein. The redox centres of cytochrome$_{aa3}$ have been localised to cytochrome oxidase subunits I and II that are present in equal stoichiometry, and Holm *et al.* (1987) have proposed that a single molecule of each of haems a and a3 is bound to each molecule of subunit I in the complex. It can be concluded that *coxI* (the gene from which transcription and translation signals for some of the mitochondrial transformation vectors have been derived) and *coxII* are expressed sufficiently to allow their protein product to accumulate to 0.026 nmoles/mg total cellular protein. This value approaches the estimates above for the maximal accumulation of CAT that is likely in chloramphenicol resistant nuclear transformants, and provides an estimate of the levels of CAT that could potentially accumulate in mitochondrial transformants expressing the chimaeric CAT genes. In practice, for CAT to accumulate at this level requires that its stability in mitochondria is similar to that of cytochrome oxidase subunits I and II, and that the chimaeric genes are expressed at rates similar to *coxI* and *coxII*; clearly it is not possible to show that these conditions would be met. Thus while no firm conclusion can be drawn about the amount of CAT that would accumulate in mitochondrial transformants, consideration of the available data clearly does not exclude the possibility that CAT could accumulate at levels equal to those in the chloramphenicol resistant nuclear transformants.

A second important consideration arising from the discussion above is that mitochondrial gene products are synthesised by expression of genes that are estimated to be present at between 100 and 250 copies per cell (Ward *et al.* 1981, Douce 1985). It is probable that after an initial transformation event the selectable gene would be present as only a single copy, and it is therefore expected that CAT would be synthesised at less than 1% of the maximal rate considered above. As mentioned in the previous section, it is still not clear how much CAT activity is required for a transformant to survive the selection procedure, but it may be relatively high, emphasising the potential importance of transforming DNA

being amplified once inside the mitochondria. As discussed in Chapter 5, studies of mtDNA recombination following protoplast fusion, of reversion of CMS cytoplasms to fertility, and of organellar mutations to drug resistance, have all provided evidence suggesting that amplification of single copy sequence is possible. In section 6.3 it was mentioned that the chloramphenicol selection system may be suited to recovering mitochondrial transformants because non-resistant cells are not killed and there is a long period for resistant lines to be segregated. The potential benefit of reducing the stringency of the selection system is also clear.

As discussed in previous chapters, design of the mitochondrial transformation strategy is hampered by lack of detailed information about many aspects of mitochondrial biology, and its assessment, by the lack of positive controls. In particular, knowledge of gene expression in plant mitochondria is not sufficient to allow a chimaeric gene to be designed with the confidence that it will be transcribed or translated in plant mitochondria, and there are no experimental systems with which to confirm or disprove the expectations. Furthermore, there is no positive control to show that expression of any such gene in transformed tissue would confer antibiotic resistance under the selection conditions used, or to show how the resistant phenotype of transformants would appear during selection. *In vitro* transcription and translation assays for plant mitochondrial genes would aid assessment of the utility of the chimaeric genes in the absence of positive controls, but none have been reported. Similarly an *in vitro* system to investigate DNA replication would facilitate the design of independently replicating vectors. Such a system is available for chloroplasts but not mitochondria (de Haas *et al.* 1987).

The lack of positive controls is inherent in a project such as this that seeks to establish a novel strategy to detect a novel event; that is, it is necessary to recover a transformed plant in order to show that the transformation vectors are selectable. However there are usually fewer unknowns in the strategies used to establish transformation in other systems. For example, establishment of transformation systems for *Chlamydomonas* chloroplasts and yeast mitochondria relied heavily on there being well established selection systems for phototrophy and respiratory competence respectively. The DNA sequences that could restore this phenotype were known, so what was required was the establishment of a DNA delivery system, in this case high velocity microprojectiles coated with DNA. Conversely, the design of chimaeric selectable markers for plant transformation relied on the established *A. tumefaciens* transformation system. After transfer to plant cells the expression of the chimaeric genes could be analysed and the levels of resistance they conferred could be determined before using them for direct selection of transformants (Herrera-Estrella *et al.* 1983a,b). For plant mitochondrial transformation there is neither an established delivery

system nor an established selection system.

Thus, it has not been possible to construct compelling arguments for or against the potential success of the strategy presented in this thesis for the recovery of plant mitochondrial transformants. Similarly, experimental systems with which to investigate many of the remaining uncertainties in the strategy are currently not available. What now remains is simply to test the strategy in transformation experiments with the mitochondrial vectors until a transformant is recovered or until it becomes evident that such transformants will be recovered so rarely as to make the system of little practical use.

8.4 Oligomycin Resistance as a Selectable Phenotype.

Early on in this project the decision was taken to adapt an established antibiotic selection system for use in mitochondrial transformation rather than to develop an entirely new selection strategy, not least of because such an alternative strategy was not readily apparent. However, of the various inhibitors of mitochondrial activity that were available, oligomycin emerged as the most promising. It binds to the F0 portion of the mitochondrial F1-F0 ATP synthase, blocking proton translocation and hence ATP synthesis. Its attraction as a selectable marker was two-fold. Firstly, the chloroplast CF1-CF0 ATP synthase was shown to be insensitive to oligomycin, remaining unaffected by concentrations of antibiotic 60 times greater than that required to impair the mitochondrial enzyme (Avron and Shavit 1965, McCarty and Racker 1967, Maury *et al.* 1981). The latter authors showed that although a chloroplast envelope K^+/H^+ pumping ATPase was inhibited in isolated chloroplasts, inhibition was not complete even at concentrations 10 times greater than is effective on mitochondria. Secondly, mutations to resistance in yeast and *Neurospora* map to the genes for either subunits 6 or 9 of the F1-F0 ATP synthase, and two independent mutations to oligomycin resistance in Chinese hamster cells have been shown to result from the same base substitution in the mitochondrial ATP6 gene (Breen *et al.* 1986).

Subunits 6 and 9 of the F0 ATP synthase are encoded in mitochondrial DNA in higher plants, and when this work was initiated, the ATP9 gene had been isolated from maize and its nucleotide sequence determined. A gene encoding an oligomycin detoxifying enzyme was not available, however the nucleotide sequence of several oligomycin resistant alleles of fungal ATP9 genes had been determined. The possibility was considered that if one of these mutations were to be introduced into *atp9* from maize it may similarly confer resistance to

Figure 8.1

Mutations to Oligomycin Resistance in *atp9* of Yeast.

The sequences of *atp9* from yeast, tobacco and maize are given in single letter code. Homology to the tobacco sequence is indicated by a vertical line between the residues. Amino acid residue substitutions that have been inferred in oligomycin resistant alleles of the yeast gene are indicated by arrows. The L to F transition has been isolated on four separate occasions. There is not sufficient similarity between the mutations or between the yeast and tobacco sequences confidently to predict substitutions that would confer resistance to the latter; interestingly, the oligomycin sensitive wildtype tobacco and maize sequences carry the alanine residue at position 21 that in yeast confers resistance. All data on resistance in the yeast genes is taken from Ooi *et al.* (1985), and the sequences of the tobacco and maize genes are taken from Bland *et al.* (1986).

oligomycin and thus provide a selectable marker for transformation.

There were however several problems inherent in this alternative strategy. Firstly, the mutations conferring oligomycin resistance to fungal ATP9 are located at several dispersed sites in *atp9*, and the similarity between the maize and fungal proteins was not sufficient to confidently predict a substitution that would confer resistance (Figure 8.1). Secondly, there was no way of experimentally verifying that an introduced mutation would lead to an oligomycin resistant F0 ATP synthase. Thirdly, there was no way to determine whether an oligomycin resistant F0 ATP synthase would be sufficient to allow plant cell growth on oligomycin; if the drug impairs other processes within the cell these would remain entirely unaffected in the transformants. Thus this approach was not considered superior to the one finally adopted because positive controls were similarly lacking.

An alternative approach involving the isolation of oligomycin resistant tobacco mutants and screening them for a mutated ATP9 gene was considered less attractive than using bacterial antibiotic resistance markers that had already been utilised for transformation of plant cell nuclei. Recently however, Aviv and Galun (1989) have isolated an oligomycin resistant cell line of *Nicotiana sylvestris* following treatment with the mutagen N-nitroso-N-methylurea. They have provided genetic evidence that the mutation is cytoplasmic, and, by analogy to some fungal mutations, have suggested that it involves *atp9* because it also confers resistance to venturicidin. This cell line, named Oli^{r}38, has been obtained from Dr. Galun and the characterisation of the oligomycin sensitivity of its mitochondria and and sequence analysis of their ATP9 gene are underway.

Should a mutation be discovered in *atp9*, this allele would be a strong candidate as a selectable marker for transforming mitochondria to oligomycin resistance. Such a scheme would have the following advantages over the strategy adopted in this thesis,

1. The initial recovery of the mutant allele provides evidence that *in vivo* the selectable marker can bestow resistance to the cell, irrespective of the effects that oligomycin may have on cellular processes.

2. The cells derived from the Oli^{r}38 can be used to show that under the conditions to be used for selection of transformants, cells expressing the mutant allele can be recovered. It should also be possible to show that the selection procedure can be used to recover new oligomycin resistant mutants following mutagenesis of wild-type tissue.

3. The selectable marker is a mitochondrial gene so one can be confident that it will be expressed in the transformants. No chimaeric gene construction is necessary.

4. The selectable marker will be homologous to the resident ATP9 gene which it could replace by double crossover or gene conversion. This

obviates the need to provide the selectable marker with its own expression signals, or sequences for maintenance.

5. Presumably, the initial mutation in Oli^{r}38 occurred in only a single copy of *atp9*, and this allele must have been amplified and expressed sufficiently to result in a resistant phenotype; this process is analogous to that which is envisaged to be necessary immediately after transformation with mitochondrial transformation vectors.

6. Integration into the mitochondrial genome by homologous recombination will not introduce additional sequence into the genome, and so avoids problems that may result from such insertions.

7. The selectable marker will almost certainly lead to resistance only if it is expressed in mitochondria. Extra specificity may be introduced by transforming cells with just that part of the gene that contains the mutation so that an intact, active, copy can be acquired only after recombination with the resident ATP9 gene.

The doubts associated with the other strategies concerning the expression, efficacy and acceptability of the selectable marker would be either resolved or avoided by the use of such a mutant mitochondrial allele. The weak point in the strategy is the difficulty in unambiguously assigning the resistant phenotype of Oli^{r}38 to a mutation that may be observed in *atp9*. However sequence analysis of several resistant and sensitive alleles should provide reliable circumstantial evidence. In addition, the spontaneous rate of mutation to oligomycin resistance in tobacco cell cultures in the absence of mutagen remains to be determined; from the results of the published chloroplast and mitochondrial transformation experiments, it probably needs to be less than 1 in 10^7 cells.

If this selectable marker is found to be effective for recovering mitochondrial transformants it will provide a suitable experimental system with which to investigate, in turn, the possibilities of introducing additional DNA sequences into plant mitochondrial genomes, of designing specific chimaeric genes for expression in mitochondria, of designing alternative maintenance strategies, and of then using these alternatives to directly select transformants.

Thus two selection strategies are proposed for mitochondrial transformation experiments; selection for either chloramphenicol resistance, over which a number of doubts remain that currently cannot be dispelled other than by achieving transformation, or oligomycin resistance which has yet to be established, but is potentially free from such doubts. Which ever is chosen, the final question to be considered concerns the DNA delivery system that is to be used to generate transformants. This is the subject of the following section.

8.5 Assessment of the Available DNA Delivery Systems.

The mitochondrial genomes of higher plant cells are separated from the cytosol by two continuous membranes that present potentially the most effective barrier to entry of transforming DNA. In contrast, the nuclear membrane may not in fact present a barrier to entry of transforming DNA (section 4.4.1), but if it does, its degeneration during meiosis potentially provides access to the nuclear genome (Meyer *et al.* 1985). Thus it seems probable that transforming DNA will be brought into contact with the mitochondrial genomes less frequently than with the nuclear genome. It could also be argued that even if the mitochondrial membranes do not exclude transforming DNA more effectively than those of the nucleus then, because the mtDNA represents less than 1% of the DNA within the cell, mitochondrial transformants might be expected to arise more rarely than nuclear transformants. However, this argument may be of little value in estimating the relative rates of transformation that can be expected for each genome; the available evidence, though circumstantial, suggests that in practice the obstacles to entry of DNA into the mitochondria or the nucleus, and the processes that may be required to overcome such obstacles and then to integrate the transforming DNA within each genome are probably different. It is these processes that will have most influence on the transformation rates and on the efficiency with which particular DNA delivery systems will generate nuclear and mitochondrial transformants. As many of these parameters probably differ for nuclear and mitochondrial transformation, the relative frequencies with which nuclear transformants are recovered using different delivery systems do not necessarily reflect the the relative frequencies with which mitochondrial transformants would be recovered.

Weising *et al.* (1988) have listed twelve different DNA delivery systems that have been successfully applied to plant cells. They have all obviously succeeded in transferring foreign DNA into the cell with at least a portion reaching the nucleus. Though these techniques were developed for nuclear transformation, with the possible exception of direct microinjection they probably do not target DNA specifically to nuclei. Similarly with the exception of microinjection and perhaps high-velocity microprojectiles and embryo imbibition, these techniques are not thought to be directly responsible for delivering DNA to the nucleus. Instead, as discussed in sections 4.4.1 and 4.4.2, it is likely that DNA is simply transferred across the plasma membrane and into the cytoplasm, with transfer to the nucleus and subsequent integration into the genome being dependent upon unknown cellular processes.

The initial step in mitochondrial transformation, as in nuclear transformation and transient expression, is transfer of DNA across the plasma

membrane. Again, with the possible exception of microprojectiles and embryo imbibition, the transformation techniques available will probably not directly transfer DNA to mitochondria, however a fraction of the DNA may subsequently be transported across the mitochondrial membrane system by processes analogous to those that mediate DNA transfer to the nucleus. Clearly, whilst the processes that may be involved in transferring DNA into mitochondria remain unknown, it will be difficult to assess the various DNA delivery systems that could be used for mitochondrial transformation. To decide which particular DNA delivery system is most appropriate, it would be necessary to know whether DNA can be transported into the mitochondria from the cytosol by a cellular process, or whether it must be delivered directly by the transformation procedure. Even if this were established, a proper evaluation of each technique would require knowledge of the quantity of DNA that is transferred, its intracellular distribution and its form (for example it could be protein bound, sequestered in vesicles, or dissolved in the cytosol). Clearly this information is not available. Despite these difficulties, given that in practice only a limited number of different transformation techniques can be used, it is important that some attempt is made to assess which will be the most efficient.

If efficiency is taken to be proportional to the number of transformants that are recovered per potentially transformable cell that is subjected to the transformation process, two alternative hypotheses can be proposed; the first is that all transformation techniques will be equally efficient at transforming mitochondria, and the second is that some techniques will be more efficient than others.

In the first instance, the number of transformants that would be recovered from any particular number of potentially transformable cells does not depend upon the DNA delivery system that is used. However the number that would be recovered in any particular experiment using each delivery system will depend upon the number of transformable cells that can be subjected to the transformation process. As the transformation frequency may be very low, the technique of choice is the one that allows the maximum number of potentially transformable cells to be treated. As discussed in Chapter 7, the most convenient way to transform large numbers of cells is by cocultivation of protoplasts or suspension cells with *A. tumefaciens*. This is not to be confused with the high efficiency of nuclear transformation normally achieved by this technique which is the usual reason for its use, but which may not be of relevance to mitochondrial transformation.

In the second instance, which is the more likely, the situation is far more complex. As mentioned above, it is probably the case that the processes responsible for efficient transfer of DNA from cytosol to nucleus differ from those that may transfer it to mitochondria. Therefore, stable nuclear transformation

rates are not necessarily indicators of the suitability of a particular technique for mitochondrial transformation. Thus, for example, although *A. tumefaciens* is able to mediate integration of T-DNA into the nuclear genomes of at least 25 % of the cells to which it is transferred (the stable transformation rate, Depicker *et al.* 1985), the mechanisms that promote nuclear transformation may preclude delivery of T-DNA to mitochondria. Conversely though only 0.01% of treated cells appear to integrate foreign DNA into their nuclear genome following PEG-calcium nitrate treatment, about 60 % of treated cells probably receive DNA (sections 4.4.1 and 4.4.2) and this may be available for uptake by their mitochondria. This may be of relevance to the results of preliminary transformation experiments reported in section 6.6 Because the frequency of nuclear transformation with the positive controls in these experiments was poor, it was considered to be premature to draw conclusions concerning the suitability of the technique for mitochondrial transformation. However, though not more than one nuclear transformant was recovered per $5x10^5$ treated cells, far more than this probably received DNA, yet in none was any of it apparently taken up by the mitochondria. Thus, if it is assumed that the selection system would have recovered mitochondrial transformants had they arisen, stable transfer of DNA from the cytosol to mitochondria does not appear to occur with greater efficiency than its integration into the nuclear genome, although it is premature to exclude a mechanism operating with a similar low efficiency.

In the absence of a clear theoretical basis on which to favour the use of any one technique, the successful transformation of yeast mitochondria using DNA coated high-velocity microprojectiles makes this the system of choice, as mitochondrial transformation has not been achieved using other techniques (Atchinson *et al.* 1980, Johnston *et al.* 1988). Many of these failed techniques presumably relied on physical principles which would also apply to procedures for transformation of plant protoplasts with purified DNA. Johnston *et al.* (1988) attribute their success in mitochondrial transformation to the delivery of DNA directly to the organelles on the microprojectiles. Mitochondrial transformation frequencies were however between 1000 and 2000 fold lower than those for nuclear transformation using high velocity DNA coated microprojectiles (Johnston *et al.* 1988, Fox *et al.* 1988).

Recently, Klein *et al.* (1988b) have reported stable nuclear transformation of *Nicotiana* leaf and tissue culture cells with the microprojectile technique. They were able to recover an average of seven transformants per bombardment, though an average of 140 cells apparently showed transient expression of the foreign DNA. If transformation of plant mitochondria by microprojectiles occurs 1000 fold less frequently than transformation of their nuclei, as with yeast cells, then about 200 bombardments would be required to recover a mitochondrial transformant. However, this calculation may be misleading as the figures for

nuclear transformation of *Nicotiana* are for stable integrations whereas those for yeast are for maintenance of independently replicating plasmids. Using alternative DNA delivery systems for nuclear transformation of yeast, transformation frequencies obtained with integrating vectors are about 1000 fold lower than those obtained with replicons capable of independent replication (Beggs 1978). It remains to be seen whether this difference exists using the microprojectile delivery system, but it is possible that stable integration of transforming DNA into the yeast nuclear and mitochondrial genomes occurs with similar frequency (about one in 10^7 cells, Hinnen *et al.* 1978). Similarly, the discrepancy between the number of plant cells that transiently express DNA and the number that are stably transformed (sections 4.4.1 and 4.4.2), suggests that integration of DNA into the nuclear genome is also an inefficient step in plant transformation. In fact, autonomously replicating viral vectors can be used to transform about 60 % of plant protoplasts following electroporation (for example Okada *et al.* 1988), which is 1000 fold greater than most claims for transformation by integration into the genome (about 0.01%, for example Fromm *et al.* 1986). The relative efficiencies with which microprojectile bombardment can be used to transfer DNA into plant cells, into their nuclei and potentially their mitochondria, remain to be determined but it is possible that considerably fewer than 200 bombardments would be required to achieve mitochondrial transformation.

The approach to mitochondrial transformation outlined above has been based upon the use of established DNA delivery systems. If alternatively one was to try and develop a system that would deliver DNA specifically to the mitochondria, the two following approaches may be most likely to succeed.

Firstly, mitochondria within a cell appear able to fuse with each other and to divide. This has been observed directly by Honda *et al.* (1966), and has been invoked to explain the recombination and exchange of mitochondrial DNA following cell fusion (Chapter 5). Furthermore, liposomes have been used to transfer DNA into plant protoplasts (Deshayes *et al.* 1985). If vector DNA was encapsulated into vesicles formed from mitochondrial membranes and these were transferred into protoplasts, it is possible that the some may fuse with the resident mitochondria and their contents may mix. Deshayes *et al.* (1985) fused the liposomes with protoplasts, so the liposomes may not have entered the cytoplasm intact but may have been destroyed by fusion with the plasmalemma, delivering the foreign DNA to the cytosol. It has been shown that chloramphenicol resistant mitochondria can become established when microinjected into cultured human cells depleted of mitochondrial DNA (King and Attardi 1988), suggesting that microinjection may provide an alternative method of delivering the DNA containing mitochondrial vesicles into plant cells. The success of this technique would rely on the production of vesicles that were

able to fuse with mitochondria in such a way as to transfer the foreign DNA to the matrix. A variation on this approach may be to introduce DNA into intact isolated mitochondria, for example by electroporation, and then to inject these back into cells.

Recently, Westweber *et al.* (1989) have shown that a short DNA molecule attached to the carboxy terminus of a protein containing a mitochondrial targeting signal can be imported into isolated yeast mitochondria. If DNA was transferred into isolated mitochondria coupled to protein import these mitochondria could perhaps be introduced back into cell to look for stable maintenance. Alternatively, it may be possible to introduce the DNA/protein complex directly into the cytosol, for example by electroporation.

At present however, the best chance of transforming mitochondria seems to involve the use of either *A. tumefaciens* or of high velocity DNA coated microprojectiles to deliver a suitable selectable marker.

CHAPTER 9.

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